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AUG 15 2012
Division of Biotechnology and GRAS Notice Review

August 14, 2012

Paulette M. Gaynor, Ph.D.
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Subject: GRAS Notification for White Mustard Essential Oil (WMEO)

Dear Dr. Gaynor:

Pursuant to proposed 21 CFR 170.36 (62 FR 18960; April 17, 1997), ConAgra Foods, Inc. hereby provides notice of a claim that the food ingredient described in the enclosed notification document is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because it has been determined to be generally recognized as safe (GRAS), based on scientific procedures, for addition to foods as an antimicrobial system for the control and prevention of sporadic recontamination.

ConAgra representatives met with the FDA on September 15, 2011, and based on the FDA's recommendation, ConAgra also met with USDA representatives on November 7, 2011, to present its case for a GRAS notification. ConAgra appreciates the feedback it received during these initial meetings, and also appreciates FDA's effort to coordinate with USDA representatives during the course of its review of this GRAS notification.

Three copies of the notification are provided as required. If you have any questions or require additional information, please do not hesitate to contact me at 202-772-4915, or ntran@exponent.com.

Sincerely,

(b) (6)
[redacted]

Nga L. Tran, Dr.PH, MPH
Principal Scientist

Cc: Steve Hermansky, PhD

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Center for Chemical Regulation and Food Safety

GRAS Determination of White Mustard Essential Oil (WMEO) For Select Food Use

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AUG 15 2012
Division of Biotechnology and GRAS Notice Review

GRAS Determination of White Mustard Essential Oil (WMEO) For Select Food Use

SUBMITTED BY:

ConAgra Foods, Inc.
6 ConAgra Drive, Mail Stop 6-460
Omaha, Nebraska 68102

SUBMITTED TO:

U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
HFS-200
5100 Paint Branch Parkway
College Park, MD 20740-3835

CONTACT FOR TECHNICAL OR OTHER INFORMATION:

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Washington, DC 20036

August 14, 2012

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List of Acronyms

4-BITC	4-benzyl isothiocyanate
4-HBITC	4-hydroxybenzyl isothiocyanate
4-OHBA	4-hydroxybenzyl alcohol
4-OHBCN	4-hydroxybenzyl cyanide
ADI	Acceptable Daily Intake
ADME	Absorption Distribution Metabolism Excretion
AITC	Allyl isothiocyanate
ALT	Alanine aminotransferase
ATSDR	Agency for Toxic Substances and Disease Registry
AUC	Area Under the blood concentration Curve
BBN	N-butyl-N-(4-hydroxybutyl)nitrosamine
BMD	Benchmark Dose
BMDS	Benchmark Dose Software
BMR	Benchmark Response
BrdU	5-bromo-2"-deoxyuridine
bw	body weight
CAS	Chemical Abstracts Service
CCRIS	Chemical Carcinogenesis Research Information System
CDC	Centers for Disease Control
CFR	Code of Federal Regulations
cfu	Colony forming units
CHL	Chinese Hamster Lung
CNS	Central Nervous System
COMET	Single Cell Gel Electrophoresis assay
DHHS	U.S. Department of Health and Human Services
DNA	Deoxyribonucleic Acid
EDI	Estimated Daily Intake
EFSA	European Food Safety Authority
EPA	U.S. Environmental Protection Agency
FAO/WHO	Food and Agriculture Organization/World Health Organization
FARE	Foods and Residues Evaluation Program
FEMA	Flavor and Extract Manufacturers Association
FDA	U.S. Food and Drug Administration
g	Gram
GMP	Good Manufacturing Practice
GRAS	Generally Recognized As Safe
h	Hour

HERA	Human and Environmental Risk Assessment
HPVIS	High Production Volume Information System
HSDB	Hazardous Substances Data Bank
IRIS	Integrated Risk Information System
IUCLID	International Uniform Chemical Information Database
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kg	Kilogram
LD50	Lethal Dose 50
LDLo	Lethal Dose Low
LOAEL	Low-Observed-Adverse-Effect-Level
m	Meter
mg	Milligram
mL	Milliliter
mm	Millimeter
MSDS	Material Safety Data Sheet
MRL	Minimal Risk Level
NCHS	National Center for Health Statistics
NHANES	National Health and Nutrition Examination Surveys
NIOSH	National Institute for Occupational Health and Safety
NOAEL	No-Observed-Adverse-Effect-Level
NTP	National Toxicology Program
°C	Degrees Celsius
OECD SIDS	Organization for Economic Co-operation and Development Screening Information Dataset
OEHHA	Office of Environmental Health Hazard Assessment
ppm	parts per million
RfD	Reference Dose
RIVM	Netherlands National Institute for Public Health and the Environment
RTECS	Registry of Toxic Effects of Chemical Substances
SCF	Scientific Committee on Food
SDH	Sorbitol dehydrogenase
TDI	Tolerable Daily Intake
TOXNET	Toxicology Data Network
TWA	Time-weighted average
U.K.	United Kingdom
U.S.	United States
USDA	U.S. Department of Agriculture
WMEO	White Mustard Essential Oil
x	Times
yrs	Years

I. GRAS Exemption Claim

A. Name and Address of Notifier

ConAgra Foods, Inc. hereby notifies the Food and Drug Administration that the use of white mustard essential oil (WMEO) described below is exempt from the pre-market approval requirements of the Federal Food, Drug, and Cosmetic Act because ConAgra Foods, Inc. has determined that such use is generally recognized as safe (GRAS) through scientific procedures.

(b) (6)

August 14, 2012

Steven J. Hermansky, Pharm D., PhD, DABT
Vice President and Fellow,
Food Protection and Regulatory Affairs
ConAgra Foods, Inc.

Date

B. Name of GRAS Substance

The name of the substance that is the subject of this GRAS determination is White Mustard Essential Oil (WMEO) containing 4-hydroxybenzyl isothiocyanate (4-HBITC, CAS #2086-86-4) that is prepared from white mustard (*Sinapsis alba)* seeds.

When white mustard seeds are ground and moistened, sinalbin is hydrolyzed to 4-hydroxybenzyl isothiocyanate (4-HBITC), a non-volatile, sharp-tasting oily compound. The reaction is catalyzed by the enzyme myrosinase that is also found in the plant and is released upon disruption of the plant membranes (E.C.3.2.3.1 thioglucoside glucohydrolase). The reaction is further activated by low concentrations of ascorbic acid.

C. Intended Use and Consumer Exposure

WMEO is proposed to be used as an antimicrobial system for the control and prevention of sporadic recontamination. This proposed use of WMEO is complementary to and not a substitution of existing process controls that ensure microbial destruction and product stability. WMEO is proposed to be added to non-carbonated beverages, ketchup, sauces/gravies in frozen meals, and egg substitutes (e.g., Egg Beaters). WMEO (containing 4-HBITC) is proposed to be added to deliver the following initial levels of 4-HBITC in foods:

- Non-carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks): 25 ppm (4-HBITC)
- Ketchup: 150 ppm (4-HBITC)

- Sauces/Gravies in frozen meals: 500 ppm (4-HBITC)
- Egg substitutes – e.g., Egg Beaters: 250 ppm (4-HBITC)

4-HBITC is unstable and hydrolyzes rapidly in an aqueous environment at neutral to basic pH values ($\geq$6). Therefore, the compounds relevant to a GRAS self-determination of the proposed uses of WMEO include the following hydrolysis or breakdown products of sinalbin: 4-hydroxybenzyl isothiocyanate (4-HBITC, CAS #2086-86-4), 4-hydroxybenzyl alcohol (4-OHBA, CAS #623-05-2), 4-hydroxybenzyl cyanide (4-OHBCN, CAS #14191-95-8), and p-cresol (CAS #106-44-5). The maximum residual levels of 4-HBITC and its primary by-products 4-OHBA, 4-OHBCN and p-cresol in foods after 5 days were predicted based on extrapolation from laboratory data and used in estimating daily intake.

The consumption of non-carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), ketchup, sauces/gravies in frozen meals, and egg substitutes (e.g. Egg Beaters) was based on food consumption records collected as part of the National Health and Nutrition Examination Surveys (NHANES) conducted in 2003-2004 and 2005-2006. The mean and 90th percentile estimated daily intake (EDI) of 4-HBITC and its hydrolyzed metabolites based on the maximum proposed use levels of 4-HBITC in non-carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), ketchup, sauces/gravies in frozen meals, and egg substitutes (e.g. Egg Beaters) were estimated on both the *per capita* and *per user* bases. At the 90th percentile *per user*, the cumulative EDIs for the US population 2+ y from all proposed food uses were 0.039, 0.21, 0.0019 and 0.0035 mg/kg bw/day for 4-HBITC, 4-OHBA, 4-OHBCN, and p-cresol, respectively.

D. Basis for GRAS Determination

ConAgra's GRAS determination for the intended uses of WMEO is based on scientific procedures as described under 21 CFR§ 170.30(b).

The intended uses of WMEO have been determined to be safe, and have also been determined to be GRAS by demonstrating that safety of intake under the proposed conditions of use is based on knowledge and information that is both publicly available and widely accepted by experts qualified by scientific training and experience to evaluate the safety of substances added to food.

Although complete safety data are not available for some of the components of WMEO, the publically available data that do exist combined with the widely disseminated knowledge concerning the chemistry of isothiocyanates and the long history of mustard consumption in general provide a sufficient basis for an assessment of the safety of WMEO for the uses proposed herein. Safe exposure limits (ADI and/or background dietary exposure) and comparisons with the EDI for each of the compounds are as follows:

4-hydroxybenzyl isothiocyanate (4-HBITC) -- A broadly based literature search revealed no safety information for 4-HBITC. Some data however were available for the closely related compound 4-BITC. The available Absorption Distribution Metabolism Excretion (ADME), short-term toxicity and mutagenicity data provide no basis for estimation of an ADI. However, the background dietary exposure to white mustard seed, based on published information on the concentration of 4-HBITC in white mustard seed and mean daily intake of mustard seed among the U.S. consumers of 1.2 g, allow an estimation of the daily intakes of 4-HBITC from mustard seed range from approximately 14.4 to 40.8 mg (0.24 to 0.68 mg/kg/day for a 60 kg individual). The *per user* 90th percentile estimate of 0.039 mg/kg bw/day from all of the proposed uses is well below background dietary exposure to 4-HBITC.

4-hydroxybenzyl cyanide (4-OHBCN) - A broadly based literature search for safety data regarding 4-OHBCN revealed only a subcutaneous LDLo in rats of 250 mg/kg. No other safety-related information was available. The *per user* 90th percentile estimate of 4-OHBCN from all proposed food uses of WMEO is 0.0019 mg/kg bw/day, and is well below the background dietary intake of 4-OHBCN, which ranges from 2.3 to 6.6 mg (0.038 to 0.11 mg/kg/day for a 60 kg individual).

4-hydroxybenzyl alcohol (4-OHBA) -- A broadly based literature search for safety-related information on 4-OHBA revealed only an *in vitro* Ames assay in S. typhimurium strain TA100 (dose range 50-2000 µg/plate), the results of which were negative. No other safety-related information was available. JECFA (2002) had no safety concerns for 4-OHBA at current levels of intake when used as a flavoring agent. As a flavoring agent, JECFA classified 4-OHBA as a structural class I. These classes of compounds were expected to be hydrolyzed to aromatic aldehydes and simple aliphatic alcohols. Based on the current intake from flavor uses, JECFA stated "no safety concern" for 4-OHBA.

Based on published information on the concentration of 4-HBITC in white mustard seed and mean daily intake of mustard seed among the U.S. consumers of 1.2 g, and conservatively assuming that all 4-HBITC formed converts entirely and exclusively to 4-OHBA, the estimated daily intake of 4-OHBA from mustard seed ranges from approximately 10.8 to 30.7 mg (0.18 to 0.51 mg/kg bw/day for a 60 kg individual). The *per user* 90th percentile estimate of 0.21 mg/kg bw/day from the proposed food uses for WMEO is within the current background dietary exposure. Given the inherent variability in the naturally occurring level of sinalbin in mustard, it is reasonable to expect that the cumulative exposure from the naturally occurring levels of 4-OHBA in the diet and the potential residual levels of 4-OHBA from all of the proposed food uses of WMEO would remain within the variable range of natural dietary exposure to 4-OHBA.

p-cresol -- The potential toxicity of p-cresol has been studied, and a safe level of exposure has been identified by government agencies and regulatory authorities such as the EPA, ATSDR, and RIVM. The available toxicology database consists of acute, subchronic, chronic and genotoxicity studies. A previously published chronic oral RfD for p-cresol (or 4-methyl phenol) has been withdrawn by EPA as a result of agency review and a new RfD is in preparation. RIVM derived a TDI of 50 ug/kg bw/day (0.05 mg/kg bw/day) based on a marginal LOAEL of 50 mg/kg bw/day for CNS effects in a 13-week study rats and application of an uncertainty factor of 1000 (10 each to account for inter- and intraspecies variability and 10 for the lack of a NOAEL). ATSDR derived an intermediate oral MRL of 0.1 mg/kg bw/day based on the results of a subchronic study in male rats and a chronic oral MRL based on results of a chronic (2-year) study in female mice (NTP, 2008) that was not available when RIVM evaluated p-cresol. In a 13-week study, the NTP (2008) identified a LOAEL of 100 mg/kg bw/day for bronchiolar hyperplasia and thyroid follicular degeneration in female mice. Data from this study were considered adequate for analysis using the benchmark dose approach for derivation of an intermediate oral MRL. The male rat data set was selected for determining the point of departure for the MRL derivation in order to provide the greatest degree of conservatism. Applying an uncertainty factor of 100 (10 for extrapolation from animals to humans and 10 for human variability) to the BMDL10 of 13.9 mg/kg bw/day yielded an intermediate-duration oral MRL of 0.1 mg/kg bw/day for *m/p*-cresol. ATSDR applied an uncertainty factor of 1000 (10 each for extrapolation from animals to humans, human variability, and use of LOAEL) to the LOAEL of 100 mg/kg bw/day for bronchiole hyperplasia of the lung and follicular degeneration of the thyroid gland in female mice for estimation of the chronic MRL of 0.1 mg/kg bw/day for *m/p*-cresol. The *per user* 90^{th} percentile estimate of 0.0035 mg/kg bw/day from all of the proposed food uses of WMEO is well below both the intermediate and chronic oral MRLs of 0.1 mg/kg bw/day.

In summary, the proposed use of WMEO provides 90^{th} percentile *per user* estimated daily intakes (EDIs) for 4-HBITC, 4-OHBA, 4-HBCN, and p-cresol for the U.S. population 2+ y (i.e., 0.039, 0.21, 0.0019, and 0.0035 mg/kg bw/day, respectively) that are either below an established regulatory benchmark (e.g., p-cresol) or at or below the current range of background dietary exposure to the compounds from consumption of mustard. All of the preclinical studies of 4-HBITC, 4-OHBA, 4-HBCN, and p-cresol have been published in the scientific literature, and therefore are generally available and are generally known among the community of qualified food ingredient safety experts. There is broad-based and widely disseminated knowledge concerning the chemistry of WMEO and its components and background dietary exposure. The data and information concerning the safety of the use of WMEO in foods (based on publicly available toxicological data regarding its constituents) as proposed in this document are also widely known and disseminated. For a food ingredient to be safe within the meaning of the FD&C Act, there must be "a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use," 21 CFR 170.3(i). The proposed use of WMEO provides 90^{th} percentile EDIs which are either below an established regulatory benchmark (e.g., p-cresol) or at or below the range of background dietary exposure to the constituents of WMEO from consumption of mustard. Therefore, following a review of all available toxicity and exposure data for WMEO and its components, it can be concluded that the proposed use of WMEO is safe within the meaning of the FD&C Act, i.e., it meets the standard of reasonable certainty of no harm.

Determination of the safety and GRAS status of WMEO for the direct addition to food under its intended conditions of use was made through the deliberation of an Expert Panel consisting of Stanley M. Tarka, Jr., Ph.D., Francis F. Busta, Ph.D , and Eric Wilhemsen, Ph.D., who reviewed a dossier prepared by Exponent as well as other information available to them. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. They individually and collectively critically evaluated published and unpublished data and information pertinent to the safety of WMEO, and unanimously conclude that the intended use of WMEO, produced consistent with cGMP and meeting appropriate specifications, as an antimicrobial in select food uses as delineated above is "generally recognized as safe" ("GRAS") based on scientific procedures.

E. Availability of Information

The data and information that serve as the basis for this GRAS determination, as well as the information that has become available since the GRAS determination, will be sent to the FDA upon request, or are available for the FDA's review and copying at reasonable times from Exponent, 1150 Connecticut Ave, NW, Suite 1100, Washington, DC 20036, telephone 202-772-4915; and email: ntran@exponent.com.

II. Description of Substance

A. Identity

White Mustard Essential Oil (WMEO) containing 4-hydroxybenzyl isothiocyanate (4-HBITC) extracted from white mustard (*Sinapsis alba)* seeds is the subject of this GRAS notification. Of the three varieties of mustard seeds, black (*Brassica nigra*), oriental (*Brassica juncea*), and white or yellow (*Sinapsis alba* or *Brassica hirta*) cultivated today, only white mustard contains the glucosinolate sinalbin (Hemingway, 1995). When white mustard seeds are ground and moistened, sinalbin is hydrolyzed to 4-hydroxybenzyl isothiocyanate (4-HBITC), a non-volatile, sharp-tasting oily compound. The reaction is catalyzed by the enzyme myrosinase that is also found in the plant and is released upon disruption of the plant membranes (E.C.3.2.3.1 thioglucoside glucohydrolase). The reaction is further activated by low concentrations of ascorbic acid.

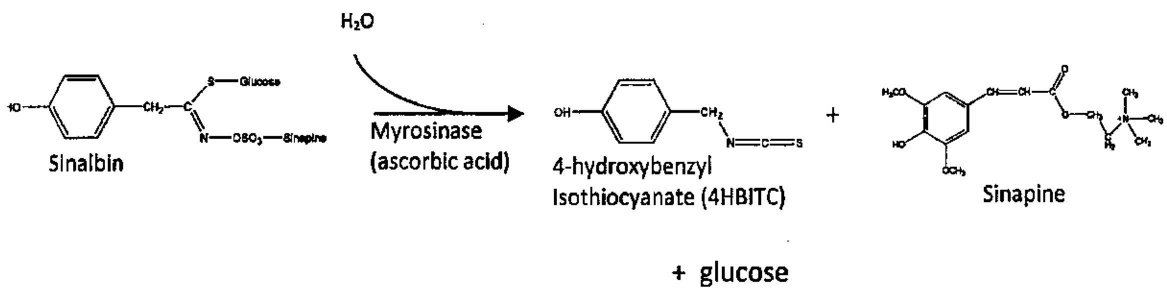


Figure 1. Hydrolysis of sinalbin to 4-hydroxybenzyl isothiocyanate

4-HBITC has wide spectrum antimicrobial properties and inactivates bacteria, yeast and fungi at relatively low concentrations. It is also unstable in aqueous systems, hydrolyzing primarily to 4-hydroxybenzyl alcohol. The stability of 4-HBITC in aqueous systems is pH dependent and is more stable in acids (few days) and less stable with increasing pH, instantaneously decomposing at alkaline pH (Buskov et al 2000).

B. Chemical and Common Names

White Mustard (*Sinapsis alba*) Essential Oil, containing 4-hydroxybenzyl isothiocyanate.

C. Chemical Abstracts Service (CAS) Registry Number

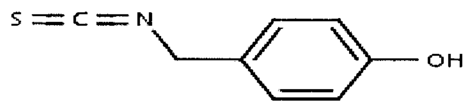
4-hydroxybenzyl isothiocyanate (4-HBITC), CAS #2086-86-4

D. Molecular Weight and Empirical and Structural Formulas

4-hydroxybenzyl isothiocyanate (4-HBITC):

Molecular formula: C8-H7-N-O-S

Molecular weight: 165.21



WMEO is composed of triglyceride and non-triglyceride components. The triglyceride component of WMEO is identical to that of the cold pressed mustard oil based on mass spectrometric analysis. The non-triglyceride compounds found in WMEO are primarily 4-HBITC and other compounds of Brassica origin. A detailed laboratory report regarding the qualitative analysis of WMEO can be found in Appendix A.

E. Production Process

WMEO is prepared as follows: white mustard seeds are first cold pressed to remove most of the fixed oil and the partially defatted mustard press cake is moistened in the presence of ethyl acetate and the activator ascorbic acid. After the reaction period, the ethyl acetate extract containing 4-HBITC and the fixed oil in the original partially defatted mustard press cake is removed by centrifugation. Low temperature evaporation of ethyl acetate under reduced pressure yields white mustard essential oil containing 4-HBITC and some of the fixed oil from the original partially defatted mustard press cake. In order to stabilize the 4-HBITC in the WMEO and also provide a means of easily dispensing it, the WMEO is mixed with maltodextrin generating an easily dispensable powdery material. It can also be diluted with a vegetable oil, dry powdered sugar or salt, for the same purpose. The manufacturing process is outlined in the following flow diagram.

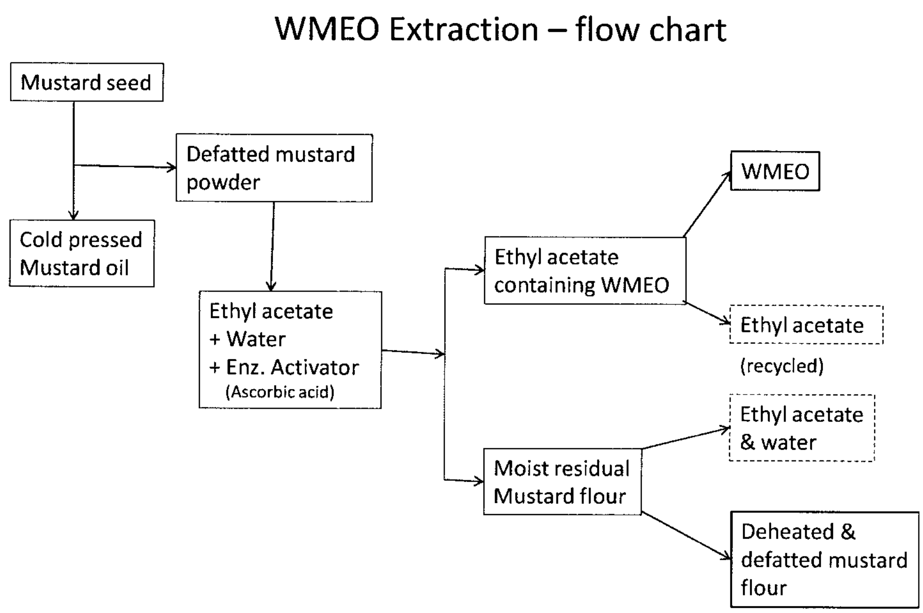


Figure 2. Flow diagram of the manufacturing process for WMEO extraction

F. Product Specifications

Specifications (of pure WMEO oil)

Common or Usual Name	White mustard essential oil (WMEO)
Botanical Name	*Sinapsis alba*
Plant Part	Seeds of *Sinapsis alba*
Appearance (Visual)	Brown oil
Country of Origin	USA, Canada or China
Solvent	Ethyl acetate/Water
Preservatives	None
4-HBITC Content	≤60% (by weight)
% Oil (total fat)	40 - 90% (by weight)
% Moisture Content (total moisture)	0 - 1% (by weight)
Residual Ethyl Acetate	< 100 ppm
Heavy Metals	≤ 10 ppm

Specifications (of stabilized WMEO in carrier)

Common or Usual Name	White mustard essential oil (WMEO)
Botanical Name	*Sinapsis alba*
Plant Part	Seeds of *Sinapsis alba*
Appearance (Visual)	Yellow powder or yellow oil
Carriers	Dry powder (maltodextrin, powdered sugar or salt) or vegetable oil; 90% (by weight)
Country of Origin	USA, Canada or China
Solvent	Ethyl acetate/Water
Preservatives	None
4-HBITC Content	≤6% (by weight)
% Oil (total fat)	4 - 9% (by weight) on dry powder carrier
	94 - 99% (by weight) in oil carrier
% Moisture Content (total moisture)	≤0.1% (by weight)
Residual Ethyl Acetate	< 10 ppm
Heavy Metals	≤ 1 ppm

Microbiological Purity

Aerobic plate count (APC)	<10 cfu/g (Method: FDA BAM, Reference: AOAC 966.23)
Mold	<10 cfu/g (Method: FDA BAM, Reference: FDA BAM: 8ed. Ch18)
Yeast	<10 cfu/g (Method: FDA BAM, Reference: FDA BAM: 8ed. Ch 18)
Coliform MPN 0-2	<0.30 MPN/g (Method: FDA BAM, Reference: AOAC 966.24)
E.coli MPN 0-2	<0.30 MPN/g (Method: FDA BAM, Reference: AOAC 966.24)
Coliform VRBA	<10 cfu/g (Method: FDA BAM, Reference BAM)
Organochloride pesticides	Meets USP requirements
Organophosphorus pesticides	Meets USP requirements
Pyrethroid pesticides	Meets USP requirements
Arsenic	$\leq$ 0.1 ppm
Cadmium	$\leq$ 0.1 ppm
Lead	$\leq$ 0.1 ppm

Certificates of Analysis, including the methods of analysis for heavy metals and pesticides for three representative lots of WMEO are provided in Appendix B.

WMEO has also been analyzed for the presence of proteins of molecular weight (MW) higher than 2.0 kD. A single lot of WMEO was extracted with ammonium bicarbonate to selectively isolate the proteins from the WMEO oil. Amino acid analyses were performed under hydrolyzed and non-hydrolyzed conditions. The average amount of total proteins (proteins and peptides) in WMEO was 11.35 ppm. A complete report of the protein characterization of WMEO can be found in Appendix C.

III. Historical Consumption and Current Regulated Uses

A. Regulated Uses

Mustard, white and yellow, are considered Generally Recognized As Safe (GRAS) by the U.S. Food and Drug Administration (FDA) for their intended use as spices, natural seasonings and flavorings per 21 CFR 182.10 and 21 CFR 582.10, and the essential oil of mustard is considered GRAS under 21 CFR 182.20 and 21 CFR 582.20.

The component 4-hydroxybenzyl alcohol (4-OHBA) that is formed from the hydrolysis and breakdown of sinalbin is also listed as a GRAS flavoring substance by the Flavors and Extract Manufacturers' Association (FEMA; FEMA No. 3987). Similarly, the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2002) had no safety concerns for 4-OHBA at current levels of intake when used as a flavoring agent. Ethyl acetate is listed as a GRAS substance for flavor adjuvants (21 CFR 182.60), and in the preparation of N-Acetyl-L-methionine (21 CFR 172.372, residue $\leq$500 ppm), modified hops extract (21 CFR 172.560, residue $\leq$1 ppm), xanthan gum (21 CFR 172.695), and sucrose fatty esters (21 CFR 172.859, residue $\leq$350 ppm). It can also be used as a secondary direct food additive (21 CFR 173.228) as a solvent in the decaffeination of coffee and tea.

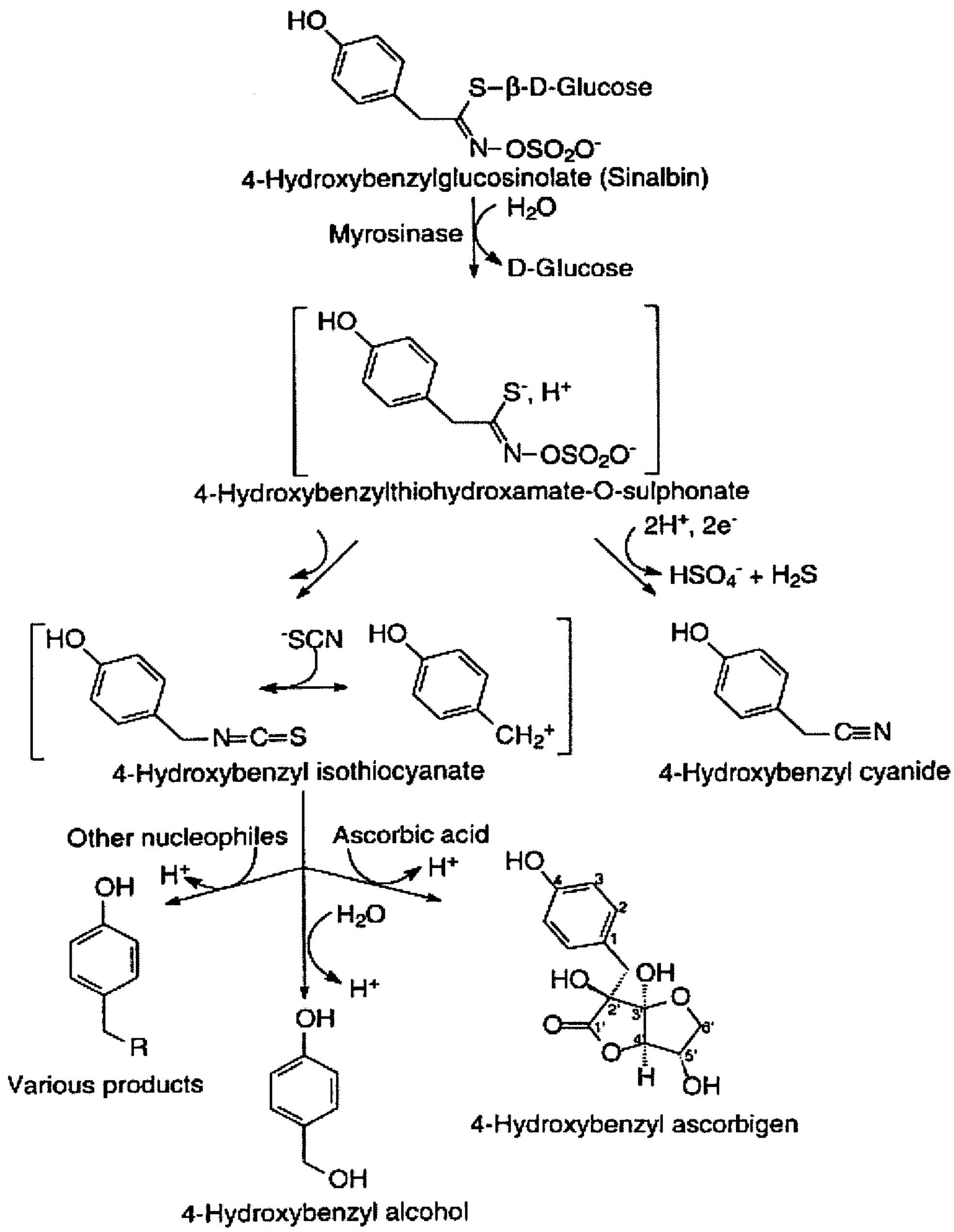
The other components of WMEO are not found in 21 CFR and have not been reviewed by JECFA or FEMA.

B. Dietary Exposure to 4-HBITC and Glucosinolate

Glucosinolates are beta-thioglucoside N-hydroxysulfates with a side chain (R) and a sulfur-linked beta-D-glucopyranose moiety (Fahey et al., 2001). The type of R group on the glucosinolate results in a wide range of biological activity and polarity for different glucosinolates. At least 120 unique glucosinolates have been identified. Many glucosinolates are found within the crucifer family Brassicaceae, though they have also been identified in other plant families of the same plant order and in other unrelated plant families (Fahey et al., 2001; Vaughn and Berhow, 2005). When plant cells are ruptured the glucosinolates present are hydrolyzed by the enzyme myrosinase, resulting in the formation of hydrolysis products including substituted isothiocyanates, nitriles, thiocyanates, epthionitriles, and oxazolidinethiones. The specific hydrolysis products produced are dependent on the plant species, R side-chain substitution on the glucosinolate, cellular pH, and cellular iron concentration (Vaughn and Berhow, 2005).

Sinalbin is the common name by which 4-hydroxybenzyl-glucosinolate is known (Fahey et al., 2001). It is the predominant glucosinolate in white and yellow mustard (Choubdar et al., 2010; Fahey et al., 2001). Hydrolysis of sinalbin by myrosinase primarily yields 4-HBITC, a non-volatile compound responsible for the hot mouth feel of mustard (Choubdar et al., 2010), and a small amount of 4-hydroxybenzyl cyanide (Buskov et al., 2000). The subsequent hydrolysis of

4-HBITC produces 4-OHBA. Structures of the glucosinolate breakdown products formed, beginning with the myrosinase-catalyzed degradation of sinalbin, are shown in Figure 3.



Source: Buskov et al. 2000

Figure 3. Structures of products formed during the myrosinase-catalyzed degradation of 4-hydroxybenzylglucosinoalte (sinalbin)

Estimates of intake of total glucosinolates have been reported for several populations. Spanish adults aged 35-64 years were estimated to consume approximately 6.5 mg glucosinolates daily (Agudo et al., 2008). In a population of German adults, men and women were estimated to consume on average 14.2 and 14.8 mg per day, respectively (Steinbrecher and Linseisen, 2009). Intake of total glucosinolates in the Netherlands was estimated to be 22 mg per day while intake was approximately 50 mg per day in the United Kingdom (U.K.) (Wattenberg et al., 1986 as cited in Agudo et al., 2008). In the United States (U.S.), the *per capita* intake of indole glucosinolates was estimated to be approximately 22.5 mg per day (Broadbent and Broadbent, 1998 as cited in Agudo et al., 2008). Overall, there is a wide range in the reported estimates of total glucosinolate intakes across different populations. This wide range of intake may be attributed to differences in the consumption of total as well as specific types of cruciferous vegetables across populations, as well as differences in dietary recall survey administration and the glucosinolate concentration data used to derive the estimates.

White mustard seed is a highly concentrated source of sinalbin. The concentration in the seed of white mustard has been reported to be approximately 29 mg sinalbin per gram of mustard seed (Ulmer and Dosdall, 2006, calculated using reported μmol/g mustard seed). The presence of sinalbin has been reported in a number of other plants (Fahey et al., 2001), though few of these plants are commonly consumed by humans. In the analysis of total glucosinolates consumed by a German population of adults (Steinbrecher and Linseisen, 2009), estimated intakes of 26 individual glucosinolates including sinalbin were calculated. The intakes of sinalbin were based on a concentration of 29.83 mg per 100 g in mustard and 14.3 mg per 100 g in watercress. Based on these concentration data, German adult men consumed an estimated mean of 0.36 mg of sinalbin daily and women consumed on average 0.17 mg of sinalbin per day.

Bunias orientalis, also known as Turkish rocket or Turkish warty rocket, was also identified as a source of sinalbin (Bennett et al., 2006). The concentration of sinalbin in young leaves from the plant was reported to be in the range of 2500 to 3000 mg of the potassium salt of sinalbin per g of fresh rocket leaves. The young stalks of Turkish rocket have been traditionally consumed raw or boiled in Russia and Romania (Turner et al., 2011). Chinese herbal remedies are also a source of sinalbin (Lee et al., 2006).

Buskov et al. (2000) demonstrated the presence of sinalbin degradation products 4-HBITC, 4-HBC and 4-OHBA in prepared and powdered mustards. The concentration of 4-HBITC and 4-OHBA were respectively determined to be approximately 0.09 mg and 0.5 mg per g of product in prepared mustard and 5.5 mg and 0.6 mg, respectively, in dry mustard powders (Buskov et al., 2000, calculated using reported μmol/g mustards). In contrast to the prepared mustard, these data indicate that 4-HBITC is the predominant form of the sinalbin degradation products (87%) in mustard powder while the 4-OHBA accounted for the balance (assuming all sinalbin originally present in the mustard was detected as 4-HBITC or 4-OHBA). Concentrations of 4-OHBCN ranged from undetectable to 0.02 mg per g prepared mustard, and from undetectable to 0.15 mg per gram of powdered mustard. It is unclear however whether these mustards were comprised solely of white mustard, or whether they contained blends of white, yellow or brown mustard powder bases.

The amount of 4-HBITC consumed as a result of consuming white mustard seed can be estimated based on intake of white mustard seed and the measured concentration of 4-HBTIC in white mustard. The concentration of 4-HBITC in white mustard seed is reported to be in the range of 12 mg per g (Indian white mustard seed) to 34 mg per g (Swedish white mustard seed) (Raghavan et al., 1971). Mean daily intake of mustard seed (from consuming foods containing mustard seed) among the U.S. consumers is 1.2 g per day based on nationwide food consumption data (NCHS 2007, 2008) and recipes that translate foods consumed into agricultural commodities (i.e. mustard seed). Assuming an average 4-HBITC concentration in white mustard seed of 23 mg per g and that mustard seed consumption in the U.S. is exclusively the white seed (also referred to as yellow mustard seed), the U.S. consumers are exposed to an average daily intake of approximately 27.6 mg of 4-HBITC (range of 14.4 – 40.8 mg).

(1.2 g mustard/day) x (23 mg 4-HBITC/g mustard) = 27.6 mg 4-HBITC

This 27.6 mg of 4-HBITC is equivalent to 167 μmoles of 4-HBITC (range of 87.3 – 247.3 μmoles).

27.6 mg 4-HBITC x (1g/1000mg) x (1 mol 4-HBITC/165 g 4-HBITC)

= 167 μmoles 4-HBITC

As the pH level of the media containing sinalbin rises from 3.5 to above 7.0, the alcohol 4-OHBA increasingly becomes the predominant hydrolysis product (Buskov et al., 2000). Assuming that all of this 4-HBITC could be converted to 4-OHBA in the presence of water (1:1 molar conversion), the potential average daily exposure to 4-OHBA from dietary intake of mustard seed in the U.S. is approximately 167 μmoles 4-OHBA (range 87.3- 247.3 μmoles). This is equivalent to 20.7 mg 4-OHBA (range of 10.8 – 30.7 mg).

167 μmoles 4-OHBA x (124 g 4-OHBA/1 mol 4-OHBA) x (1000 mg/1 g) = 20.7 mg 4-OHBA

As depicted in Figure 3, ascorbic acid may be consumed during the breakdown of 4-HBITC. Padayatty et al. (2003) have described the food sources of vitamin C as being primarily of fruit and vegetable origin found sparsely in prepared food items cooked prior to consumption. Thus, the contribution to total dietary intake of vitamin C from the foods included in this GRAS self-determination is small. Even if the prepared meal consumption were followed immediately by consumption of a vitamin C rich food, the trace residual amount of 4-HBITC remaining (<10ppm) would not have a biologically meaningful impact on total dietary vitamin C consumption. Any residual 4-HBITC would be further hydrolyzed in the digestive tract preventing any effect on total dietary vitamin C intake. In summary, the net effect of residual 4-HBITC on ascorbic acid stability in complex food systems would be quantitatively insufficient to impact vitamin C consumption patterns.

In addition to 4-HBITC, 4-hydroxybenzyl cyanide (4-OHBCN) may also be produced from the hydrolysis and breakdown of sinalbin, albeit at much lower levels. This is due to the fact that hydrolysis produces both 4-HBITC and 4-OHBCN, but not from the same molecule, and according to the data of Buskov et al., a much greater portion of sinalbin goes on to form 4-

HBITC rather than 4-OHBCN. Specifically, any molecules of the sinalbin precursor that are committed to formation of 4-HBITC, do not form 4-OHBCN (Figure 3). The hydrolysis of sinalbin by myrosinase produces a relative molar distribution of approximately 10% 4-OHBCN at pH 3.5 and up to 20% at pH 7.5 in the presence of ascorbic acid. Without added ascorbic acid, the relative molar distribution of 4-OHBCN ranges from approximately 1% up to 10%.

According to Buskov et al. (2000), the relative molar distribution of 4-HBITC formed from the hydrolysis of sinalbin by myrosinase can be nearly 100% depending on hydrolysis conditions such as pH. It can therefore be assumed that the number of moles of 4-HBITC formed (12 to 34 mg, or 73 μmoles to 206 μmoles per gram mustard seed) is approximately representative of the total number of moles of all hydrolysis products, the distribution of which will vary dependent on reaction conditions.

(0.012g 4-HBITC) x (1 mole 4-HBITC/165 g 4-HBITC) = 73 μmoles 4-HBITC

(0.034g 4-HBITC) x (1 mole 4-HBITC/165 g 4-HBITC) = 206 μmoles 4-HBITC

The data of Buskov et al. (2000) demonstrate that the maximum percent molar distribution of 4-OHBCN formed from the hydrolysis of sinalbin is approximately 20%, resulting in the possible formation of 14.6 to 41.2 μmoles, or 1.9 to 5.5 mg of 4-OHBCN per gram of mustard seed.

(20% 4-OHBCN) x (73 μmoles total products) = 14.6 μmoles 4-OHBCN

(14.6 μmoles 4-OHBCN) x (133 g 4-OHBCN/1 mole 4-OHBCN) = 1.9 mg 4-OHBCN

Based on the mean daily intake for the U.S. consumers of 1.2 g of mustard seed, this corresponds to an intake range of 2.3 to 6.6 mg of 4-OHBCN, or an average daily intake of 4.5 mg 4-OHBCN.

Based on published reports of the concentration of 4-HBITC in white mustard seed and a 1.2 g mean daily intake of white mustard seed among the U.S. consumers, it is reasonable to conclude that the daily mean intake of 4-HBITC from mustard seed ranges from roughly 14.4 to 40.8 mg while the daily mean intake of 4-OHBA from mustard seed ranges from 10.8 to 30.7 mg and the daily mean intake of 4-OHBCN ranges from 2.3 to 6.6 mg.

IV. Intended Use and Estimated Intakes

A. Intended Use

White mustard essential oil (WMEO) is a natural antimicrobial that can be used to control potential pre- or post- process low level microbial contamination (Delaquis & Mazza, 1995). Consumer food products are typically subjected to a validated kill step to ensure microbial destruction and product stability. However, in some rare and unforeseen situations, after the kill step and prior to final packaging, products may be susceptible to adventitious recontamination with microorganisms from the manufacturing environment. Additionally, some packaged meals that are not-ready-to-eat (NRTE), must be cooked thoroughly prior to consumption for assurance of food safety and quality. Some meal components that comprise prepared and packaged foods may not be fully cooked and may occasionally introduce spoilage or pathogenic microorganisms. In addition, during assembly these products may be susceptible to microbial recontamination from the manufacturing environment.

There is a need in the food industry for microbial intervention strategies targeted at reducing the risk of occurrence of the above scenarios. One of the potential solutions is the application of antimicrobial systems for the control and prevention of sporadic recontamination. There is an unmet yet clearly articulated consumer preference for use of safe natural antimicrobials over persistent chemical preservatives that are commonly used in foods. The use of natural antimicrobials serves as a nimble hurdle in addition to full compliance to industry accepted best practices such as GMP, employee hygiene, etc.

Use of mustards as natural antimicrobials has been well established (Ekanayake et al., 2006). Among the mustards, white or yellow mustard has the lowest sensory interference at the effective level of usage for proper efficacy against spoilage and pathogenic microorganisms. The active component in WMEO is 4-hydroxybenzyl isothiocyanate (4-HBITC). 4-HBITC is not persistent and is relatively unstable in aqueous environments found in common foods. The antimicrobial efficacy of 4-HBITC against common spoilage and pathogenic microorganisms is a potential solution for control of pre- and post- process contamination, and for reducing risk in assembled prepared and packaged foods. WMEO in a suitable food compatible carrier can be used as an antimicrobial ingredient in the formulation and assembly of these food products (Ekanayake et al., 2006).

WMEO is proposed to be used as an antimicrobial system for the control and prevention of sporadic recontamination. This proposed use of WMEO is complementary to and not a substitution of existing process controls that ensure microbial destruction and product stability. The efficacy data to support the intended effect of the proposed use of WMEO are detailed in Appendix D. WMEO is proposed to be added to non-carbonated beverages, ketchup, sauces/gravies in frozen meals, and egg substitutes (e.g., Egg Beaters). WMEO (containing 4-HBITC) is proposed to be added to deliver the following initial levels of 4-HBITC in foods:

- Non-carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks): 25 ppm (4-HBITC)
- Ketchup: 150 ppm (4-HBITC)
- Sauces/Gravies in frozen meals: 500 ppm (4-HBITC)
- Egg substitutes – e.g., Egg Beaters: 250 ppm (4-HBITC)

4-HBITC is unstable and hydrolyzes rapidly in an aqueous environment at neutral to basic pH values ($\geq$6). Based on the maximum proposed use levels of WMEO in foods the maximum residual levels of 4-HBITC and other hydrolysis compounds were predicted based on extrapolation from laboratory data. Details of the laboratory data and extrapolation methods are summarized below.

Laboratory Data

ConAgra conducted four hydrolysis experiments in buffer systems at pH 3.5, 6.0 and 9.0, and one experiment in Roasted Red Bell Pepper puree (pH 4.97) as a representative of food matrix. The levels of 4-HBITC, 4-OHBA, 4-OHBCN and p-cresol were determined initially, and at set intervals thereafter by ethyl acetate extraction followed by high performance liquid chromatography (HPLC) identification with quantitation using internal standards. Results were reported for 4-HBITC, 4-OHBA, 4-OHBCN and p-cresol at pH 3.5, 6.0 and 9.0 for up to 120, 96, and 48 hours, respectively. In the pH 4.97 pepper puree matrix, data for 4-HBITC were recorded for up to 72 hours. Figures corresponding to these data are summarized and discussed below. The complete laboratory study is provided in Appendix A.

Extrapolation Methods and Assumptions to Predict Residues in Foods

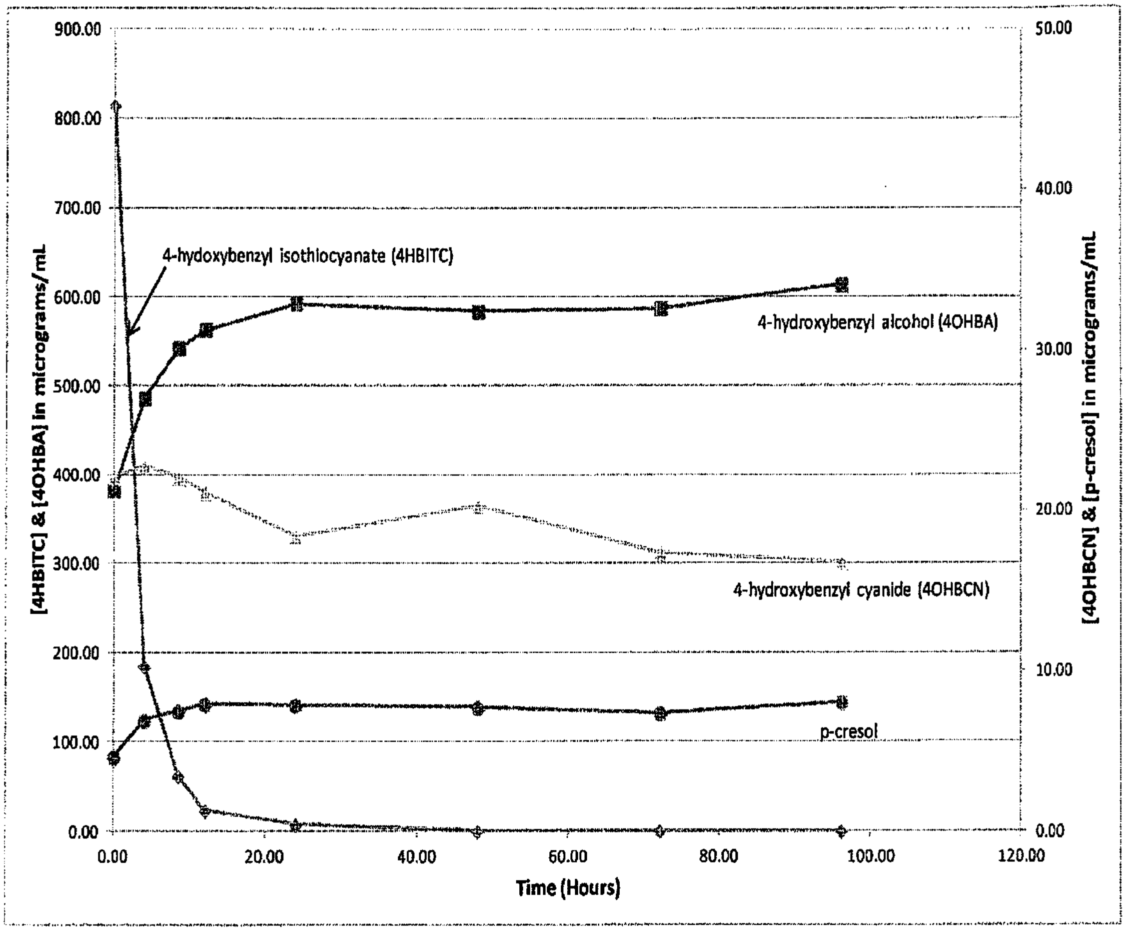
Residual levels of WMEO component hydrolysis and breakdown products in food are dependent on the pH of the food matrix and the duration of WMEO being incorporated in the matrix. Analytical data obtained for the concentrations of the hydrolysis products demonstrate that concentrations of these products tend to be smoothly varying functions of the parent concentration, pH and time. Thus, in order to determine the levels of 4-HBITC, 4-OHBA, 4-OHBCN and p-cresol in food matrices that contain added WMEO for which data points are unavailable, an extrapolation can be made using the available data. The following data extrapolations were conducted:

1) **Linear Extrapolation to Durations and pH Ranges Not Measured in Laboratory Setting:**

   pH 3.5: none needed – data were available for up to 120 hours (5days).

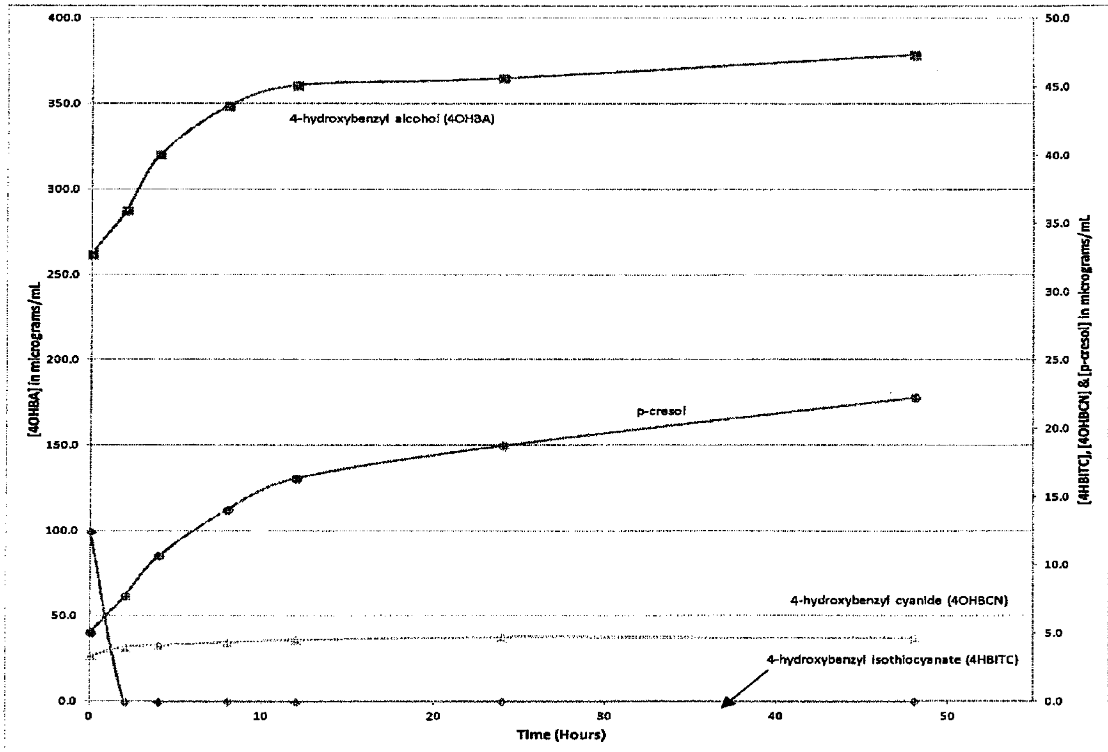
   pH 6.0: data were available for up to 96 hours (4 days), with the parent compound (4-HBITC) being completely hydrolyzed at 48 hours. The levels of the metabolites (4-OHBA, 4-OHBCN, p-cresol) increase over time, and appear to plateau at 72 hours (see Figure 22 from ConAgra report below). Thus, the maximum measured level reported between 0-96 hours were assumed at t = 120 hours for these 3 metabolites.

Figure 22. Concentration of 4HBITC, 4OHBA, 4OHBCN and p-cresol with time, pH 6.0, 21°C. Average of 4 determinations per time point.



pH 9.0: Data were available for up to 48 hours (2 days), with the parent compound (4-HBITC) being completely hydrolyzed within 2 hours. The levels of the metabolites (4-OHBA, 4-OHBCN, p-cresol) increase rapidly during the first 8 hours and appear to plateau thereafter (see Figure 24 from ConAgra report below).

Figure 24. Concentration of 4HBITC, 4OHBA, 4OHBCN and p-cresol with time, pH 9.0, 21°C. Average of 4 determinations per time point.



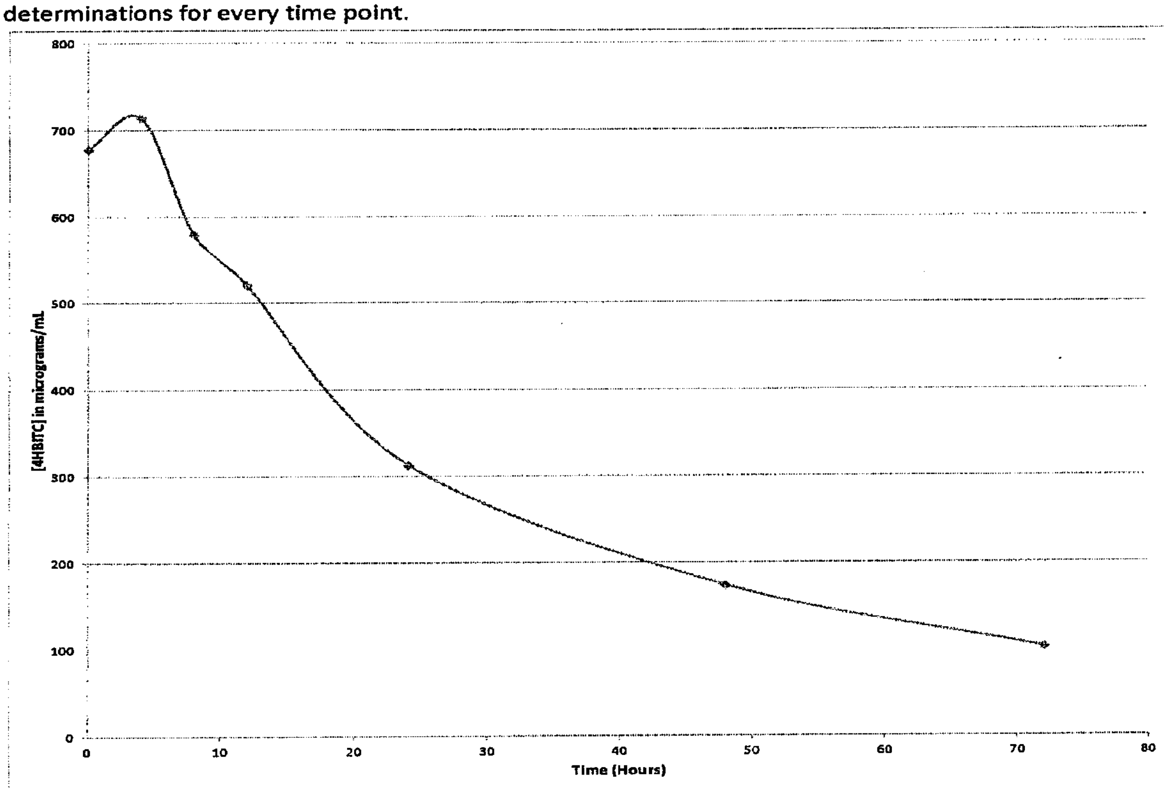
A slope and intercept were derived based on the linear interpolation between t=12 hours and t=48 hours, and metabolite levels at t=72, 96 and 120 hours were predicted by extrapolation based on the derived slope and intercept (Table 1).

Table 1. Linear Interpolation at pH 9

	4-OHBA	4-OHBCN	p-cresol	
Slope	0.51	0.003	0.16	linear interpolation between t=12 hours and t=48hrs
Intercept	353.90	4.54	14.57	linear interpolation between t=12 hours and t=48hrs
t (hours)	**Remaining metabolite level in µg/mL at t time (starting 4-HBITC concentration 1000ug/mL)**			
72	390.67	4.75	26.27	Extrapolated
96	402.93	4.82	30.17	Extrapolated
120	415.19	4.89	34.08	Extrapolated

pH 4.96 (roasted red bell pepper puree): Data for 4-HBITC were available for up to 72 hours (3 days). 4-HBITC level appears to decline linearly on the log scale (see Figure 27 from ConAgra report below).

Figure 27. Concentration of 4HBITC in Roasted Red Bell Pepper puree, pH 4.97, 21°C. Average of 4 determinations for every time point.



A slope and intercept were derived based on the log linear interpolation between t=0 hours and t=48 hours, and 4-HBITC was predicted at t=96 and 120 hours based on the derived slope and intercept.

Table 2. Log Linear Interpolation at pH 4.96 (Roasted Red Bell Pepper Puree) and Predicted 4-HBITC Levels

Time (hrs.)	4-HBITC (µg/mL)	4-HBITC (m mol/L)	LN[4-HBITC]
		slope	-0.03
		intercept	1.44
72	90.50	-0.6024	1.8265
96	49.53	0.300	-1.20
120	25.55	0.155	-1.87

The levels of the metabolites (4-OHBA, 4-OHBCN, p-cresol) were not measured/reported in this experiment. Values for these metabolites at pH 4.97 were interpolated based on measured levels at pH 3.5, 6 and 9 (see above). The values for these metabolites predicted to be associated with pH 4.96 (roasted red bell pepper puree) are summarized in Table 3 below.

Table 3. Predicted Levels of 4-HBITC By-Products at 72, 96 and 120 hours at pH 4.97 (Roasted Red Bell Pepper Puree)

Time (hrs)	4-OHBA (mmol/L)	4-OHBCN (mmol/L)	p-cresol (mmol/L)	4-OHBA µg/mL	4-OHBCN µg/mL	p-cresol µg/ml
72	4.02	0.06	0.05	499.28	7.49	5.81
96	4.29	0.05	0.07	532.23	7.28	7.30
120	4.44	0.06	0.07	551.31	8.26	7.91

2) Application of Laboratory Data to Foods to which WMEO is Intended to be Added:

To apply the laboratory testing results to a food matrix with a pH different from the tested pH, the following assumptions were made to ensure that the maximum levels of parent compound (4-HBITC) and its hydrolysis products would be predicted at day 5 after treatment:

a) Parent compound: 4-HBITC

- If the food product *minimum* pH is ≤4, then the level data (µg/mL) from the testing done at pH = 3.5 will be used.
- If the food product *minimum* pH is >4 and ≤5, then the level data from testing done at pH = 4.97 (roasted bell pepper puree) will be used.
- If the food product minimum pH is >5 and ≤ 6, then the level data from testing done at pH = 6 will be used
- If the food product minimum pH is >6, then the level data done at pH = 9 will be used.

Table 4. Summary of pH Rules Used to Apply Laboratory Data to Foods, 4-HBITC

Food Minimum pH	Corresponding pH from Laboratory Data
≤4	3.5
>4 - ≤ 5	4.97 (roasted bell pepper puree)
>5 - ≤ 6	6
>6	9

Rationale: 4-HBITC hydrolyzes at a fast rate in aqueous environment at high pH (>6) and less rapidly at lower pH (<6). Hence, making a correlation between the *minimum* pH in food to the *lower* tested pH provides inherent conservatism to assure that the *maximum* residual 4-HBITC is used in the dietary exposure assessment.

b) Hydrolysis by-products: 4-OHBA, 4-OHBCN, and p-cresol

- If the food product *maximum* pH is ≤4, then the level data (μg/mL) from the testing done at pH = 3.5 will be used.
- If the food product *maximum* pH is >4 and ≤ 6, then the level data from testing done at pH = 6 will be used
- If the food product *maximum* pH is >6, then the level data done at pH = 9 will be used.

Table 5. Summary of pH Rules Used to Apply Laboratory Data to Foods, 4-OHBA, 4OHBCN, and p-cresol

Food Maximum pH	Corresponding pH from Laboratory Data
≤4	3.5
>4 - ≤ 5	4.97 (roasted bell pepper puree)
>4 - ≤ 6	6
>6	9

Rationale: 4-HBITC hydrolyzes at a fast rate in aqueous environment at higher pH values (≥6). Hence, making a correlation between the *maximum* pH in food to the tested pH provides inherent conservatism to assure that the *maximum* levels of the products from the hydrolysis of 4-HBITC are used in the dietary exposure assessment.

Predicted Maximum Residual Levels in Foods 5 Days

Based on the laboratory data, which was tested at a starting level of 1000 μg/mL for the parent compound 4-HBITC, the maximum residual levels of 4-HBITC and its hydrolysis products can be predicted in food at day 5 at their corresponding pH (Table 6). Scaling to the maximum proposed use levels of WMEO in foods, the maximum residual levels of 4-HBITC and its hydrolysis products are predicted at day 5 and summarized in Table 7.

Table 6. Predicted Maximum Residual 4-HBITC, 4-OHBA, 4-OHBCN, and p-cresol Levels in Foods at 120 hours Based on Laboratory Tested Starting Level of 1000 μg/mL 4-HBTIC

	Food pH		Level (ppm) @ 120 hours			
Product	**min**	**max**	**4-HBITC**	**4-OHBA**	**4-OHBCN**	**p-cresol**
Non- carbonated beverages	3	4	100.00	550.80	4.94	7.37
Ketchup	3.7	3.8	100.00	550.80	4.94	7.37
Sauce: frozen meat component including gravy	4.0	7.0	100.00	415.19	4.89	34.08
Egg substitutes – e.g., Egg Beaters	8	8	0.00	415.19	4.89	34.08

Table 7. Predicted Maximum Residual 4-HBITC, 4-OHBA, 4-OHBCN, and p-cresol Levels at 120 hrs Based on Maximum Proposed Use Levels of 4-HBITC in Foods

Product	Food pH Range	Maximum Proposed Use Level of 4-HBITC (ppm)	Scaling factor	Predicted Maximum Residual Levels (ppm)			
				4-HBITC	4-OHBA	4-OHBCN	p-cresol
Non-Carbonated Beverages	3 – 4	25	0.025	2.50	13.77	0.12	0.18
Ketchup	3.7-3.8	150	0.15	15.00	82.62	0.74	1.11
Sauces and gravy in Frozen Meal	4-7	500	0.5	50.00	207.59	2.45	17.04
Egg substitutes – e.g., Egg Beaters	8	250	0.25	0.00	103.80	1.22	8.52

The predicted maximum residual levels of 4-HBITC and its primary by-products 4-OHBA, 4-OHBCN and p-cresol in foods after 5 days in Table 7 are used in the dietary exposure assessment in support of the safety assessment of the proposed use of WMEO in non-carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), ketchup, sauces/gravies in frozen meals, and egg substitutes (e.g. Egg Beaters).

With the exception of non-carbonated beverages, food products will be consumed at least 5 days (approximately 120 hours) after being manufactured. In the case of non-carbonated beverages, it is possible that time to market and, therefore, consumption of product may be of shorter duration than 5 days. That is, during occasional times of extremely high product demand or product shortages, non-carbonated beverages may be delivered to market and consumed in less than 5 days and sometimes in as short as 24 to 48 hours after manufacture. However, in the vast majority of cases, the time between manufacture and consumption will significantly exceed 5 days and, generally, greatly exceed this time. Based upon this information, all products were evaluated for consumption with an assumption that consumption occurs 5 days after manufacture.

B. Estimated Daily Intake (EDI)

The consumption of non-carbonated beverages, ketchup, sauces/gravies in frozen meals, and egg substitute (e.g. Egg Beaters) was based on food consumption records collected as part of the National Health and Nutrition Examination Surveys (NHANES) conducted in 2003-2004 and 2005-2006. This continuous survey is a complex multistage probability sample designed to be representative of the civilian U.S. population (NCHS 2007, 2008). The NHANES datasets provide nationally representative nutrition and health data and prevalence estimates for nutrition and health status measures in the United States. The NHANES survey over-samples minorities, low-income groups, adolescents ages 12-19 years, and adults 60 years of age and older. Statistical weights are provided by the National Center for Health Statistics (NCHS) for the surveys to adjust for the differential probabilities of selection. As part of the examination, trained dietary interviewers collect detailed information on all foods and beverages consumed by respondents in the previous 24 hour time period (midnight to midnight). A second dietary recall is administered by telephone 3 to 10 days after the first dietary interview, but not on the same day of the week as the first interview. The dietary component of the survey is conducted as a partnership between the U.S. Department of Agriculture (USDA) and the U.S. Department of Health and Human Services (DHHS). DHHS is responsible for the sample design and data collection, and USDA is responsible for the survey's dietary data collection methodology, maintenance of the databases used to code and process the data, and data review and processing. A total of 16,783 individuals in the survey period 2003-2006 provided 2 complete days of dietary recalls.

Using the NHANES consumption data, Exponent estimated 2-day average intake of non-carbonated beverages (energy, sport drinks, fruit-flavored drinks; fruit juice; and juice drinks), ketchup, sauces/gravies in frozen meals, and egg substitutes (e.g., Egg Beaters) for the U.S. population 2 years and older. The list of food codes included in the analysis is provided in Appendix D of this report. The analysis was limited to individuals who provided two complete and reliable dietary recalls as determined by NCHS. The 2-day average intakes by each individual were estimated using Exponent's Foods and Residues Evaluation Program (FARE™ version 8.64) software. Exponent uses the statistically weighted values from the survey in its analyses. The statistical weights compensate for variable probabilities of selection, adjust for non-response, and provide intake estimates that are representative of the U.S. population. Exponent estimated the daily intake on a "*per user*" basis. In this analysis, a "user" is anyone who reported consuming a food on either of the survey days. We identify each individual who reported consuming a food on either of the survey days, and we use that individual's responses for both survey days. Zero consumption days are included in calculating that individual's average daily intake. For example, if someone reported consuming 200 grams of coffee on day 1 and 150 grams of coffee on day 2, his/her 2-day average coffee consumption would be 175 grams ([200+150]/2).

The 2-day average intake of foods from the NHANES 2003-2006 were combined with the predicted maximum residual levels of 4-HBITC and its primary hydrolysis by-products (4-OHBA, 4-OHBCN and p-cresol) in foods after 5 days in non-carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), ketchup, sauces/gravies in frozen meals, and egg substitutes (e.g. Egg Beaters) (see Table 7) to derive the estimated daily intake (EDI) of the residual 4-HBITC and its major hydrolysis by-products.

The mean and 90th percentile EDI of residual 4-HBITC and its hydrolysis by-products based on the maximum proposed use levels of 4-HBITC in ketchup, egg substitutes (e.g., Egg Beaters), non-carbonated beverages and sauces from frozen meals were estimated on both the *per capita* and *per user* bases and summarized in Table 8. At the 90th percentile *per user*, the cumulative EDIs from all proposed food uses were 0.039, 0.21, 0.0019 and 0.0035 mg/kg bw/day for 4-HBITC, 4-OHBA, 4-OHBCN, and p-cresol, respectively.

Table 8. Estimated Daily Intake of 4-HBITC, 4-OHBA, 4-OHBCN, and p-cresol; US 2+, NHANES 2003-06; 2-day average

Food Groups	Compound	Maximum Level @ 120 hr (ppm)*	*Per Capita* (mg/kg bw/d)		*Per User* (mg/kg bw/d)	
			Mean	90th	Mean	90th
Ketchup	4-HBITC	15.00	0.0010	0.0030	0.0035	0.0077
	4-OHBA	82.62	0.0055	0.0164	0.0193	0.0421
	4-OHBCN	0.74	0.0000	0.0001	0.0002	0.0004
	p-cresol	1.11	0.0001	0.0002	0.0003	0.0006
Egg substitutes – e.g., Egg Beaters	4-HBITC	0	0	0	0	0
	4-OHBA	103.80	0.0007	0	0.0926	0.2274
	4-OHBCN	1.22	0.0000	0	0.0011	0.0027
	p-cresol	8.52	0.0001	0	0.0076	0.0187
Energy, sport drinks, fruit-flavored drinks	4-HBITC	2.5	0.0031	0.0102	0.0162	0.0342
	4-OHBA	13.77	0.0170	0.0563	0.0893	0.1883
	4-OHBCN	0.12	0.0002	0.0005	0.0008	0.0017
	p-cresol	0.18	0.0002	0.0008	0.0012	0.0025
Fruit juice	4-HBITC	2.50	0.0043	0.0124	0.0120	0.0250
	4-OHBA	13.77	0.0239	0.0682	0.0663	0.1374
	4-OHBCN	0.12	0.0002	0.0006	0.0006	0.0012
	p-cresol	0.18	0.0003	0.0009	0.0009	0.0018
Juice drinks	4-HBITC	2.50	0.0025	0.0087	0.0124	0.0257
	4-OHBA	13.77	0.0137	0.0481	0.0681	0.1415
	4-OHBCN	0.12	0.0001	0.0004	0.0006	0.0013
	p-cresol	0.18	0.0002	0.0006	0.0009	0.0019
Sauces from frozen meals**	4-HBITC	50.00	0.0006	0	0.0325	0.0533
	4-OHBA	207.59	0.0025	0	0.1347	0.2211
	4-OHBCN	2.45	0.0000	0	0.0016	0.0026
	p-cresol	17.04	0.0002	0	0.0111	0.0181
All foods combined	**4-HBITC**	- -	**0.0115**	**0.0311**	**0.0167**	**0.0389**
	4-OHBA	- -	**0.0634**	**0.1690**	**0.0916**	**0.2104**
	4-OHBCN	- -	**0.0006**	**0.0016**	**0.0008**	**0.0019**
	p-cresol	- -	**0.0011**	**0.0026**	**0.0015**	**0.0035**

*Based on maximum proposed use level of WMEO;
**An average sauce portion from frozen meals was determined to be 33% based on a subset of the frozen meal foods identified to contain sauce.

V. Safety Data

A. Introduction

A comprehensive search of the literature bearing on the toxicity of WMEO and its components and/or hydrolysis products was first conducted in September 2010. Databases and websites that were searched included PubMed, Toxicology Data Network (TOXNET), Hazardous Substances Data Bank (HSDB), ChemIDplus, Organization for Economic Co-operation and Development Screening Information Dataset (OECD SIDS), U.S. Environmental Protection Agency (EPA; including Integrated Risk Information System (IRIS), High Production Volume Information System (HPVIS)), U.S. Food and Drug Administration (FDA), Regulations.gov, Human and Environmental Risk Assessment (HERA), Agency for Toxic Substances and Disease Registry (ATSDR), National Toxicology Program (NTP), JECFA, European Food Safety Authority (EFSA), and the California Toxicity Criteria Database. Search terms included (4-hydroxybenzyl isothiocyanate or 2086-86-4), (4-hydroxybenzylcyanide or 4-hydroxybenzyl cyanide or 14191-95-8), (4-hydroxybenzyl alcohol or 623-05-2), and (p-cresol or 4-methylphenol or 106-44-5) and (toxicity or toxic or safety or safe* or hazard* or animal* or human or in vitro or in vitro or diet or consumption or acute or chronic). The compounds relevant to a GRAS self-determination of the proposed uses of WMEO include the following by-products of sinalbin: 4-hydroxybenzyl isothiocyanate (4-HBITC, CAS #2086-86-4), 4-hydroxybenzyl alcohol (4-OHBA, CAS #623-05-2), 4-hydroxybenzyl cyanide (4-OHBCN, CAS #14191-95-8), and p-cresol (CAS #106-44-5). A literature search update was performed June 11, 2012, to locate any information relevant to the safety of WMEO and breakdown products that had been published since the September 2010 literature search was performed. Search terms and databases searched were the same as those used for the initial literature search. The literature search update captured 3 publications, all concerning *in vitro* effects of p-cresol.

B. Safety Data for WMEO Components

4-hydroxybenzyl isothiocyanate (CAS # 2086-86-4)

A broadly based literature search including PubMed revealed no safety information for 4-HBITC. Some data however are available for the closely related compound 4-benzyl isothiocyanate (4-BITC). 4-BITC is identical to 4-HBITC except that it lacks a para-hydroxy group. Both compounds are expected to have similar endogenous properties and reactivity, however of the two molecules, 4-HBITC is predicted to be more rapidly conjugated and excreted owing to the presence of the hydroxyl functional group (Casarett and Doull 1996). 4-BITC is therefore a conservative surrogate for 4-HBITC. The available toxicity data for 4-BITC are summarized below.

Absorption, Distribution, Metabolism and Excretion

Mercapturic acid formation appears to be the major route for metabolism of 4-BITC. In rats, oral administration of 4-BITC resulted in detection of the conjugate mercapturic acid in 20-hour pooled urine at a level of 62% of the administered dose (Brusewitz et al., 1977). In guinea pigs and rabbits, which do not excrete mercapturic acids as readily as humans or rats, the cyclic mercaptopyruvic acid conjugate was detected following oral administration of 4-BITC (Gorler et al., 1982). Metabolism in humans is expected to be most similar to that in rats (Gorler et al., 1982; Brusewitz et al., 1977) and to result primarily in mercapturic acid formation and excretion.

Compared to 4-BITC, 4-HBITC contains a hydroxyl group at the para- position of the benzyl moiety. This structural difference would not be expected to alter mercapturic acid formation at the thiocyanate moiety of 4-HBITC as compared to 4-BITC. The only anticipated effect of the presence of the hydroxyl group would be possible additional metabolism through glucuronidation at the hydroxyl group, followed by similar excretion pathways as the mercapturic acid.

The generation of thiocyanate anion (-SCN, see Figure 1) is possible from either 4-HBITC or 4-BITC, however it is not a safety concern. The thiocyanate anion is of low acute toxicity with an approximate LD_{50} in rats of 750 mg/kg bw (Hansson et al., 2008) and historical consumption of foods containing glucosinolates indicates that the thiocyanate breakdown product is safe. All glucosinolates share a common mechanism of breakdown resulting in the formation of thiocyanate anion, among other molecules (Fenwick and Heaney 1983, Fahey et al. 2001, Holst and Williamson 2004). For example, the glucosinolates sinigrin and 4-hydroxyglucobrassicin from *Brassica juncea* (oriental mustard) and gluconapin, progoitrin, 4-hydroxyglucobrassicin, and 4-methoxyglucobrassicin from *Brassica napus* (canola) have all been demonstrated to produce thiocyanate anion upon breakdown of the parent glucosinolates found in the plant seeds (Hansson et al 2008). Foods with broad historical consumption that are understood to contain thiocyanate anion as a result of the breakdown of glucosinolates include broccoli, cabbage, Brussels sprouts, cauliflower, kale, collards, kohlrabi (Stoewsand 1995), canola (Hansson et al., 2008), arugula, radish, capers, wasabi and others (Fahey et al. 2001).

Foods containing glucosinolates, and thus isothiocyanates and thiocyanate anion, are recognized as healthy and safe, and have in fact been investigated for their potential health benefits (Fahey et al. 2001, Kwak and Kensler 2010). Evidence suggests that consumption of fruits and vegetables, especially cruciferous vegetables, which are known to contain glucosinolates, is linked to the reduction of incidences of certain types of cancer (Fahey et al. 2001). Two servings of vegetables per day may result in as much as a 50% reduction in cancer risk (for certain sites) and this activity is largely believed to result from dietary components, in particular, glucosinolates. For example, broccoli sprouts, which contain even higher levels of glucosinolates than mature broccoli (Fahey et al. 2001), have been tested in clinical trials for efficacy in decreasing biomarkers associated with liver cancer (Kensler et al. 2005 and 2012, Egner et al. 2011). These disease prevention mechanisms appear to act through the anti-oxidant response element promoter region of DNA to enhance transcription of genes involved in anti-oxidant defenses, and detoxification of electrophiles (Kwak and Kensler 2010).

Acute Toxicity (Animals)

The acute effects of BITC, the major metabolite of 4-BITC, and several other compounds were investigated in the urinary bladder of female Fisher 344 rats (Masutomi et al., 2001). BITC, allyl isothiocyanate (AITC), or BITC-metabolites conjugated either with glutathione, cysteinylglycine, cysteine, or the major metabolite of BITC, the mercapturic acid, were instilled intravesically into the empty bladders of the rats. Bladders were exposed to BITC at a dose of 2.8 mg/kg or the same mol quantity (37 µmol/kg) of BITC-metabolites for 2 hours. Nineteen hours thereafter, the animals were intravenously administered 5-bromo-2"-deoxyuridine (BrdU) and killed 1 h later. Bladders were excised and fixed. In the majority of the rats tested, BITC instillation resulted in epithelial vacuolar degeneration, focal regions of mucosal apoptosis/necrosis, diffuse mucosal desquamation, inflammatory infiltration and edema, and in 2 out of 6 rats, focal mucosal or submucosal hemorrhage. In contrast, the mercapturic acid metabolite of BITC showed little to no changes in these markers of tissue damage. BrdU labeling for all groups was essentially dependent on the degree of cytotoxic potential of each compound. The authors postulate that the results of this study support the idea that if it exists, BITC-derived carcinogenicity may be a result of the cytotoxic effects of unconjugated BITC in the urine. However, the data from the study indicate that the major metabolite of BITC (i.e., mercapturic acid) undergoes urinary excretion and is not toxic to the bladder epithelium.

Short-Term Toxicity (Animals)

The effects of 4-BITC on bladder physiology were investigated in a short-term feeding study by Akagi et al. (2003). F344 weanling rats of both sexes were fed a diet containing 1000 ppm 4-BITC. In the first study group, urinary pH, sodium, potassium and chloride were measured at days 1 and 7 of feeding. Differences were noted in urinary sodium and chloride levels at day 7, but not in pH or potassium. Because of the very short duration of the study it is unclear whether these differences would persist, however by day 7 urinary chloride and sodium exhibited a trend toward recovering to control levels. In the next experiment, the thickness of the urinary bladder epithelium of rats fed the 1000 ppm diet was assessed. The thickness of the epithelium increased from day 1 of the feeding through day 7 and was statistically significant compared to controls. It should be noted however, that only 6 points were recorded per rat to assess bladder epithelial thickness and these points were manually selected, possibly limiting the quantitative power of this assay. Other parameters were assessed in the same rats including bladder inflammation, vacuolation, erosion and apoptosis/necrosis. In females only (n = 5 per group), on day 7 inflammation was noted in 3 out of 5 rats. All other noted parameters in this segment of the study either nearly or completely recovered to control levels by day 7, repeating the trend seen in the urinary levels of chloride and sodium. Finally, BrdU labeling was conducted on the epithelia from male and female rats on the test diet. Labeling indicated a clear statistically significant increase in cell proliferation of the bladder epithelium especially on day 2, followed by an obvious trend towards recovery on day 7, albeit still significantly different than control measures. The pattern of initial changes in the bladder upon the introduction of 1000 ppm 4-BITC into the diet followed by actual recovery or a trend towards recovery of measured parameters summarizes the finding of this study. The authors assert that these changes may play an early role in urinary bladder carcinogenesis induced by 4-BITC, however literature searches conducted in support of this GRAS determination failed to identify any studies which revealed cancer as a result of oral administration of 4-BITC. 4-BITC did however show signs of ability to promote urinary bladder carcinogenesis, however only following a 4 week initiation period with 500 ppm of a nitrosamine bladder carcinogen administered through the drinking water (Okazaki et al., 2003).

The short-term toxicity of 4-BITC was investigated by Lewerenz et al. (1992) in a 4-week study in weanling male Wistar rats. Rats were dosed daily by gavage with 0, 50, 100, or 200 mg/kg bw/day of 4-BITC. No treatment related deaths were observed, however one rat died due to gavage error. Total body weight gain was decreased in all treatment groups compared to control which received vehicle only (sunflower oil). Food intake differed between some treated groups and control during the study, but not at the end of the study. At day 28, body weights were significantly different for all treatment groups compared to the control group. At the conclusion of the study, rats treated at 200 mg/kg bw/day had decreased mean corpuscular hemoglobin, total white blood cell count, and lymphocytes and increased neutrophils. Statistically significant changes in hematological parameters were not noted in the other groups. Urinary parameters measured at the conclusion of the study (4 weeks) indicated increased serum cholesterol in all treatment groups and decreased serum triglycerides in the 200 mg/kg bw/day treatment group only. Differences in urine volumes were noted at points during the study. However, statistically significant differences were not reported at the conclusion of the study, and a dose-response effect was not noted. Pathological examination of the 200 mg/kg bw/day group revealed decreased absolute organ weights in all organs examined except the adrenals. Relative organ weights were increased except for thymus and spleen which were decreased, and heart, which showed no significant change. Some, but not all of these changes were noted in the 100 mg/kg bw/day dose group, and the 50 mg/kg bw/day group showed statistically significant increases in thyroid, liver, and adrenal weights. The authors note that changes in organ weights may have been driven by the decreases in body weight. In 6 animals of the 200 mg/kg bw/day and 1 animal in the 100 mg/kg bw/day dose groups, peritoneal inflammation was observed. Histological examination revealed proliferation of the bile duct epithelium in all treatment groups. In the highest dose group, there was cloudy swelling of the hepatocytes. The authors indicated that renal dysfunction may be a mechanism of action based on decreased urine volume in the 200 mg/kg bw/day dose group and increased urinary protein in the 100 and 200 mg/kg bw/day groups at the 2 and 3 week time-points. Additionally, lactate dehydrogenase, another indicator of kidney function, was increased at 3 weeks in all treatment groups, but the significance of this was unclear given the lack of a dose- response effect, with the low dose of 50 mg/kg bw/day displaying the greatest increase compared to control.

Effects were noted for at least some observed parameters at all dose levels in the Lewerenz study and the authors did not explicitly establish a no-observed-adverse-effect-level (NOAEL) or low-observed-adverse-effect-level (LOAEL). In their discussion, Lewerenz et al. noted that it was not possible to determine whether some of the observed changes were due to secondary effects of BITC toxicity and not related to any particular mechanism of action of the compound. In particular, the authors proposed that the decreased relative weights of the thymus and spleen and the histological changes in the mesenteric lymph nodes in the highest treatment group were suggestive of an immune response to chemical intoxication itself and not particular to the effects of 4-BITC.

Carcinogenesis (Animals)

In a cancer promotion and initiation study, six-week old male F344 rats were given 500 ppm N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) in their drinking water for 4 weeks as an initiation treatment, then fed a basal diet alone or supplemented with 100 or 1000 ppm BITC, with or without simultaneous 25 ppm BBN in the drinking water for 36 weeks post-initiation (Okazaki et al., 2003). A second analogous group of rats received the same 36 weeks of treatment, but did not receive BBN initiation. In the uninitiated BITC groups treated at 1000 ppm in the diet, all animals (10) were observed to have both simple and papillary nodular hyperplasia, while only one animal from the 10 ppm BITC group had simple hyperplasia. No carcinomas were observed in the BITC treated groups without initiation, however in the initiated groups BITC appeared to promote bladder carcinogenesis.

In another study by Okazaki et al. (2002) the promoting potential of BITC was demonstrated in the urinary bladder of male rats following oral administration at a dose of 0.1% in the diet (1000 ppm) for 32 weeks. Without initiation, however, no carcinomas were observed in the bladder for the group treated with BITC alone, only one incidence of epithelial hyperplasia was observed at the 100 ppm treatment but 10/10 rats had papillary nodular hyperplasia in the 1000 ppm group. Neoplastic lesions were noted in the livers of rats receiving the initiating treatment (diethylnistrosamine followed by BBN). However BITC treatment in the diet decreased the incidence of these lesions. Based on these observations, the authors concluded that BITC might have carcinogenic potential (not definitive as a potential promoter) however it was a "potent" chemopreventive agent against bladder cancer.

In another study designed to test the promotion potential of BITC for bladder and liver carcinoma, weanling F344 male rats were treated with initiating carcinogen followed by attempted tumor promotion with BITC or PEITC (Hirose et al., 1998). BITC treatment alone without initiation led to slightly increased observations of hyperplasia of the urinary bladder epithelium, however no carcinomas were observed. BITC treatment following initiation did however appear to promote bladder carcinoma as evidenced by an increased number of carcinoma lesions observed in the initiated BITC-treated group. BITC treatment alone however did not result in any carcinoma or adenoma of the liver, and initiated animals treated with BITC had decreased adenoma and carcinoma burden compared to control initiated animals that did not receive BITC, demonstrating the potential chemopreventive properties of BITC.

Reproductive and Developmental Toxicity (Animals)

In a study of pregnancy outcomes related to 4-BITC, female Sprague-Dawley rats were dosed daily by gavage either pre- (days 1-5 of gestation) or post- (days 7-13 of gestation) implantation with 0, 12.5, 25 or 50 mg 4-BITC/kg bw/day (Adebiyi et al., 2004). No vaginal bleeding was observed in any treatment groups and no delivery complications were noted, in contrast to the suggestions of the *in vitro* isolated uterine tissue experiments previously reported (Adebiyi et al., 2003). In the highest dose group, 2 maternal deaths were recorded in the pre-implantation treatment group and 1 death was recorded in the post- implantation group immediately following treatment. Gross observations associated with treatment included hypo-activity, perinasal staining, piloerection, and hunched posture in the pre-implantation group, and hypo-activity, lethargy, ruffled fur, perinasal staining and hunched posture in the post-implantation group. There were decreases in body weight recorded during the treatment phase (gestation days 1-5) for the 25 and 50 mg/kg bw/day pre-implantation groups. However following this, weight gain ensued at a rate similar to that of the control and low dose treatment group. Rate of weight gain differed in the post-implantation group, resulting in statistically significant weight differences in the two highest dose groups at 15 days, but these differences were not statistically significant at day 20. No significant differences were observed in relative organ weights in either the pre- or post-implantation groups. Observed fetal resorptions were not statistically significant for either the pre- or post- implantation groups. There were not significant differences in the number of viable fetuses as a result of treatment, and gross observations revealed no important findings. Pups of mothers treated with 25 and 50 mg/kg bw/day however, had significantly decreased body weights (up to ~25% less), and placental weights. The authors concluded that 4-BITC did not cause significant pre- or post- implantation fetal loss, but that the slightly decreased fetal and placental weights could be of importance. They noted decreased placental weights at all dose levels and decreased fetal weights at the 25 and 50 mg/kg bw/day doses of 4-BITC and indicated that this is possibly due to maternal toxicity. Numerous authors have reviewed the association between observations of maternal toxicity (due to decreased food intake and weight decrement) and their relevance/contribution to reproductive and teratogenic effects (Khera, 1985; WHO 1987).

The Netherlands National Institute for Public Health and the Environment (RIVM) has evaluated the non-cancer oral toxicity data for thiocyanic acid and derived a tolerable daily intake (TDI) of 0.011 mg/kg bw/day (Baars et al., 2001). This TDI was also based on the NOAEL of 0.11 mg/kg bw/day for thyroid effects. Additionally, Dahlberg et al. (1984) reported a NOAEL of 0.11 mg/kg bw/day for thyroid effects in humans for thiocyanic acid (thiocyanate, -SCN and HSCN, CAS # 463-56-9). However, based on the metabolism of 4-BITC described above, thiocyanate is not expected to be generated *in vivo* from 4-BITC or 4-HBITC metabolism, despite the depiction of thiocyanate in the myrosinase catalyzed degradation scheme of sinalbin presented in Figure 3, which is based on *in vitro* reactions that are not conducted under biological conditions. Specifically, these reactions are not carried out in the presence of glutathione, which reacts rapidly and nonenzymatically with 4-BITC at the isothiocyanate moiety (Gorler et al., 1982; Brusewitz et al., 1977), effectively preventing the release of thiocyanate ion. Although components of thiocyanate are present in the isothiocyanate moiety of 4-BITC and 4-HBITC, the structure of the major metabolites of 4-BITC indicate that 4-BITC does not release thiocyanate *in vivo*, and by extension, 4-HBITC is also not anticipated to release thiocyanate.

Reproductive and Developmental Toxicity (*in vitro*)

Adebiyi et al. (2003) studied the effects of a papaya extract containing 4-BITC as the primary phytochemical, on functional and pathological parameters of isolated uterine tissue. Uterine strips from gravid and non-gravid rats were bathed in appropriate buffers and treated with various concentrations of the extract. The extract inhibited prostaglandin- and oxytocin-induced contractions. Recovery of contractions was weak, and at higher doses, irreversible. Light microscopy revealed degeneration of the endometrium and myometrium with cytoplasmic vacuolation. It is not clear that the effects noted in this *in vitro* model can be extrapolated *in* *vivo*. In this model, the compound was applied in solution directly to the isolated uterine tissues. As such, the model lacks endogenous metabolic capacity and does not reflect oral exposure to 4-BITC.

Mutagenicity

4-BITC proved mutagenic in the Ames assay (Yamaguchi 1980 as cited in Masutomi et al., 2001), was positive for chromosomal aberration (Musk and Johnson, 1993) and caused deoxyribonucleic acid (DNA) strand breaks in the COMET assay (Musk et al., 1995). However, 4-BITC has also been observed to result in cell cycle arrest (Zhang et al., 2006) and suppression of metastasis potential in cancer cell models (Wu et al., 2010), events that are considered anti-carcinogenic. These discrepancies are explained by the detoxification which occurs via glucuronidation *in vivo* but not *in vitro*. Indeed, the genotoxic effects of 4-BITC were markedly reduced in *in vitro* experiments conducted in biological media (Kassie et al., 1999). In summary, the i*n vitro* data on both the mutagenic and chemopreventive properties of 4-BITC, and isothiocyanates in general, are conflicting.

4-hydroxybenzyl cyanide (4-OHBCN; CAS No. 14191-95-8)

A broadly based literature search including PubMed revealed only a subcutaneous lethal dose low (LDLo) in rats of 250 mg/kg (Nishie and Daxenbichler, 1980 as cited in ChemIDplus, 2010). No other safety-related information was available.

The presence of a cyanide moiety in 4-OHBCN and potential in vivo generation of free cyanide during the hydrolysis of 4-HBITC is not of concern for many of the same reasons that the production of thiocyanate anion is not a concern during glucosinolate breakdown. Conjugated cyanides (formally called nitriles) are known to be generated from a broad array of glucosinolates (Thornalley, 2002), and glucosinolates from a variety of commonly consumed plants sources share a common breakdown mechanism, producing metabolites containing a cyanide moiety similar to 4-OHBCN. The safe historical human consumption of a variety of plants from the *Brassica* family including broccoli, cabbage, Brussels sprouts, cauliflower, kale, collards, kohlrabi (Stoewsand 1995), canola (Hansson et al., 2008), arugula, radish, capers, wasabi and others (Fahey et al. 2001) indicates that consumption of compounds containing a cyanide moiety in the diet and in the context presented herein is safe. Furthermore, glucosinolate-containing vegetables and plants have been widely recognized not for any toxic effects but rather for their health benefits, in particular chemoprevention (Fahey et al. 2001). Therefore, in spite of a considerable body of literature associated with the toxicological mechanistic action of the cyanide ion and related compounds, these chemical moieties will not be further reviewed in this GRAS self-determination due to the common occurrence of these compounds in the human diet from various sources.

4-hydroxybenzyl alcohol (4-OHBA; CAS No. 623-05-2)

A broadly based literature search including PubMed revealed the results of an *in vitro* Ames assay in S. typhimurium strain TA100 (dose range 50-2000 μg/plate) with 4-OHBA, the results of which were negative (CCRIS, 1993). No other safety-related information was available.

JECFA (2002) had no safety concerns for 4-OHBA at current levels of intake when used as a flavoring agent. The Committee evaluated a group of flavoring agents comprised of 46 structurally related substances. The structural feature common to all members of the group was a primary oxygenated functional group bound directly to a benzene ring which also contained a hydroxy or alkoxy substitute. As a flavoring agent, 4-OHBA was classified by JECFA as a structural class I compound. These classes of compounds are expected to be hydrolyzed to aromatic aldehydes and simple aliphatic alcohols. The intake of 4-OHBA in humans is 6 μg/day in Europe and in the United States it is 0.06 μg/day (JECFA, 2002). JECFA stated that the intake of 4-OHBA does not exceed the threshold for structural class I compounds, which is 1800 μg/day. Based on the current intake, JECFA (2002) stated 4-OHBA has "no safety concern".

p-cresol (CAS No. 106-44-5)

Absorption, Distribution, Metabolism and Excretion

The phenolic compound p-cresol is metabolized primarily by glucuronidation, and due to its small size, excreted in the urine (Lesaffer et al., 2003). There is some indication from studies presented below, that route of administration of p-cresol has toxicokinetic implications (ATSDR 2008). There are no studies comparing the toxicokinetics of cresols following dietary and gavage administration, but there is information for a related chemical, phenol. Phenol toxicity following oral gavage dosing is different than following administration in the drinking water. In the case of phenol, there are data that suggest that toxicity is correlated with peak blood concentration rather than with total dose, such as the area under the blood concentration curve (AUC) following a single gavage dose or repeated daily doses. This is consistent with data from Bray et al. (1950, as cited in ATSDR 2008), who observed that *p*-cresol was more toxic when given by stomach tube to fasting rabbits than when the rabbits were given their daily food 1–2 hours before dosing with *p*-cresol; suggesting that *p*-cresol became mixed with the food, which delayed its absorption. Also relevant is a study by Morinaga et al. (2004, as cited in ATSDR 2008), which found concentrations of free cresols in liver and spleen from rats given a single oral gavage dose much higher than in blood at all times after dosing (up to 8 hours). Based on these observations and the fact that an oral gavage exposure protocol does not resemble human environmental exposure scenarios to cresols, only dietary studies are considered for Minimal Risk Level (MRL) derivation, even though some LOAELs by gavage are lower than dietary LOAELs (ATSDR 2008).

Distribution of p-cresol was studied in rats by Morinaga et al. (2004, as cited in ATSDR 2008). Conjugated and unconjugated cresols were determined in tissues at set time-points up to 8 hours following gavage administration of a mixture of m- and p-cresols. Unconjugated concentrations in the brain, lung, and muscle for both cresols were similar to those in the blood. Glucuronidated cresols were highest in the kidney, followed by the liver. Glucuronidation appeared to predominate as the metabolic route, as sulfate conjugates were also less than glucuronide conjugates in tissues in general, and especially in the liver and kidneys. The sulfate of m-cresol appeared at slightly higher concentrations for all tissues compared to the p-cresol sulfate, indicating slightly different metabolic disposition for the two forms. It should be noted that proportions of the conjugates formed from metabolism of simple phenols such as cresols can vary with dose and species (ATSDR 2008). As noted in the study by Morinaga et al., (2004, as cited in ATSDR 2008), rats preferentially metabolize m-cresol to the sulfate, and p-cresol to the glucuronide.

As indicated by the distribution studies, cresols are largely metabolized to sulfate and glucuronidate conjugates and excreted in the urine (ATSDR 2008). Rabbits administered an oral dose of cresols eliminated 60-72% of the administered dose via the urine as glucuronide conjugate, whereas only 10-15% of the dose was recovered as sulfate (Bray et al., 1950, as cited in ATSDR 2008). In very early studies, Williams similarly described the observation of 14.5 – 23.5% of an administered dose to rabbits being excreted in the urine as sulfate conjugate (1938, as cited in ATSDR 2008). A small percentage (~3%) of o- and m-cresol may also be hydroxylated, however this does not occur with p-cresol (Bray et al., 1950, as cited in ATSDR). Rather, both free and conjugated p-hydroxybenzoic acid were detected in the urine. In total, only 1-2% of the administered dose was detected in the urine unconjugated.

Further studies using rat liver slices coupled with *in vitro* methods indicate that quinones or other oxidative species may be formed during p-cresol metabolism (Thompson et al., 1994, 1995, 1996, and Yan et al., 2005, both as cited in ATSDR 2008). According to the metabolic scheme proposed by Yan et al. (2005, as cited in ATSDR 2008), the quinone metabolites and intermediates are ultimately glutathionylated. It should be noted that regardless of the ability to detect these intermediates and alternate metabolites in *in vitro* systems, the sulfate and glucuronate conjugates of cresols account for approximately 10 – 23.5% and 60 – 72%, respectively, of the administered oral dose and are excreted within 24 hours following intake (Bray et al., 1950, and Williams 1938, both as cited in ATSDR 2008).

Acute Toxicity (Animal)

Effects of cresol administered by oral gavage are markedly different than those observed in dietary studies (ATSDR 2008). Administration of cresols by oral gavage to animals resulted in lowest observed-adverse-effect levels (LOAELs) much lower than LOAELs defined in dietary studies. For example, LD50 values for undiluted cresols in rats ranged from 121 to 242 mg/kg (EI du Pont 1969, as cited in ATSDR 2008), whereas dietary doses in the range of 1,000–2,000 mg/kg bw/day for intermediate durations caused little or no toxicity in rats and mice (NTP 1992b, ATSDR 2008). Serious neurological effects (i.e., lethargy, tremors, convulsions) were seen in rats dosed by oral gavage with doses ranging from 450 to 600 mg/kg bw/day for 90 days (EPA 1988b, 1988c, 1988d; TRL 1986; Tyl 1988a, 1988b, as cited in ATSDR 2008), but no such effects were observed in the dietary studies at much higher dose levels (NTP 1992a, 1992b, 1992c, 2008, ATSDR 2008). The reason for this difference is not known, but it is most likely related to differences in toxicokinetics between the two methods of cresol administration.

- No acute-duration oral MRL was derived for cresols due to lack of acute dietary exposure studies (ATSDR 2008). An MRL is defined as an estimate of daily human exposure to a substance that is likely to be without an appreciable risk of adverse effects (noncarcinogenic) over a specified duration of exposure. MRLs are derived when reliable and sufficient data exist to identify the target organ(s) of effect or the most sensitive health effect(s) for a specific duration within a given route of exposure. MRLs are based on noncancerous health effects only and do not consider carcinogenic effects.

Short-Term Toxicity (Animal)

Evaluation of the results of the available intermediate-duration dietary studies by ATSDR (2008) indicated that the most sensitive target was the nasal respiratory epithelium of rats and mice dosed with *p*-cresol or a mixture of *m*- and *p*-cresol (NTP 1992b, ATSDR 2008). The effects occurred in male and female rats and mice dosed for 28 days or 13 weeks. The data sets considered by ATSDR for MRL derivation were the 28-day experiment in female rats and the 13-week experiment in male rats based on the lowest effect levels identified in both sets, 95 mg/kg bw/day in the 28-day experiment and 123 mg/kg bw/day in the 13-week experiment. In the 28-day study, the incidences of hyperplasia of the nasal respiratory epithelium in female rats dosed with 0, 27, 95, 268, 886, and 2,570 mg/kg bw/day of *m/p*-cresol were 0/5, 0/5, 3/4, 5/5, 5/5, and 5/5, respectively. In the 13-week study, the incidences in male rats dosed with 0, 123, 241, 486, 991, and 2,014 mg/kg bw/day *m/p*-cresol were 0/10, 3/10, 8/10, 10/10, 8/10, and 10/10, respectively. The latter series was preferred because of the longer duration of exposure and because of the increased reliability of a dose-response curve based on 10 rats per group rather than on only 5 rats per group in the 28-day study.

In the principal study for the MRL, groups of Fischer 344 rats (20/sex/group) were administered *m/p*-cresol (58.5% *m*-cresol, 40.9% *p*-cresol) in the diet at levels of 0, 1,880, 3,750, 7,500, 15,000, or 30,000 ppm for 13 weeks (NTP 1992b, ATSDR 2008). The corresponding doses of test compound estimated by the investigators were 0, 123, 241, 486, 991, and 2,014 mg/kg bw/day for males and 0, 131, 254, 509, 1,024, and 2,050 mg/kg bw/day for females. End points evaluated included clinical signs, food consumption, organ weights, clinical chemistry and hematology, and gross and microscopic appearance of organs and tissues. Although the dose groups consisted of 20 rats of each sex, 10 males and 10 females were used for clinical chemistry, hematology, and urinalysis studies and the remaining 10 rats/sex/group were used in gross pathology, organ weight, and histopathological studies. There were no deaths during the study. Final body weight in the 2,014/2,050 mg/kg bw/day males and females was reduced 17 and 12%, respectively, relative to controls. Food consumption was also reduced (about 10%) in this group during the first week of the study. Additionally, males and females in this group exhibited rough hair coat; females also had a thin appearance. Absolute and relative liver weights were significantly increased (11–12%) in males at 486 mg/kg bw/day and in females at 1,024 mg/kg bw/day. Absolute and relative kidney weight was increased in males at 991 mg/kg/day. In general, hematology findings were unremarkable, although there was a tendency to hemoconcentration at 2,014/2,050 mg/kg bw/day early in the study. Clinical chemistry tests showed an increase in serum alanine aminotransferase (ALT) in males and females exposed to 2,014/2,050 mg/kg bw/day and in sorbitol dehydrogenase (SDH) in males at 2,014 mg/kg bw/day only on day 5.

Bile acids in serum were increased in females at 2,050 mg/kg bw/day on day 90 and at 241 and 991 mg/kg bw/day in males also on day 90. There was no indication of renal injury as judged by the results of urinalyses. Significant histopathological changes included minimal bone marrow hypocellularity in males and females (likely secondary to decreased weight gain) at 2,014/2,050 mg/kg bw/day, and increased colloid (minimal) in thyroid follicular cells in females at 509 mg/kg bw/day and in males at 15,000 ppm (991 mg/kg bw/day). An increased dose-related incidence and severity of hyperplasia and glandular hyperplasia of the nasal respiratory epithelium was observed in male and female rats. Severity was minimal at 123/131 mg/kg bw/day, mild at 486/509 mg/kg bw/day, and moderate at 2,014/2,050 mg/kg bw/day. The lesions were located at the most anterior portions of the nasal septum, dorsal arch, and medial aspect of the nasal turbinates. The hyperplasia was characterized by increased number of goblet cells and pseudogland formation due to the infolding of the hyperplastic cells. The hyperplastic areas were associated with single cell necrosis. The incidences in males dosed with 0, 123, 241, 486, 991, and 2,014 mg/kg bw/day were 0/10, 3/10, 8/10, 10/10, 8/10, and 10/10, respectively. A similar trend was seen in female rats, but 3/10 control females also exhibited hyperplasia (3/10, 1/10, 5/10, 9/10, 8/10, and 10/10 at 0, 131, 254, 509, 1,024, and 2,050 mg/kg bw/day, respectively).

The most comprehensive study of cresols by dietary exposure was conducted by the National Toxicology Program (NTP 1992b, ATSDR 2008). In that study, each individual isomer and an *m/p*-cresol mixture were tested in rats and mice for 28 days; in addition, *o*-cresol and *m/p*-cresol were tested in rats and mice for 13 weeks. Assessing the comparative toxicity of the cresol isomers, NTP (1992b) noted that: "In general, there were no significant indications of distinct toxicities between the three isomers." However, nasal lesions only occurred in rats and mice dosed with *p*-cresol and *m/p*-cresol in the 28-day studies and in rats and mice dosed with *m/p*-cresol in the 13-week studies. Since *m*-cresol alone was not tested in the 13-week studies, it is unknown whether longer dietary exposure to this isomer would produce similar lesions. Thus, it would appear that *p*-cresol is the most toxic of the isomers with regard to inducing nasal lesions and, since no other significant toxicities were observed in these dietary studies, the MRL for *m/p*-cresol should also be protective for exposures to the individual cresol isomers. Therefore, the intermediate- and chronic-duration oral MRLs for *m/p*-cresol also can be adopted for *o*-, *m*-, and *p*-cresol.

Data from the NTP (1992b) were considered adequate for analysis using the benchmark dose approach for MRL derivation (ATSDR 2008). Benchmark dose models in the EPA Benchmark Dose Software (BMDS) (version 2.0) were fit to the incidence data for nasal lesions in male and female rats exposed to *m/p*-cresol in the diet for 13 weeks in order to determine potential points of departure for the MRL. Comparing fits across nine different models, the log-logistic model was determined to be the best-fitting model for the male rat data set, whereas the quantal linear model was the best-fitting model for the female rat data set. Following EPA's Benchmark Dose Guidance (EPA 2000a, as cited in ATSDR 2008) to select a point of departure, a benchmark response (BMR) of 10% was selected for the benchmark analysis of nasal lesion incidence data in the 13-week NTP (1992b) study. The benchmark dose (BMD) corresponding to a BMR of 10% extra risk was 55.89 mg/kg bw/day. BMDL10s (i.e., 95% lower confidence limits on the model-estimated dose associated with a 10% extra risk for nasal lesions) calculated with the best-fitting models for each data set were 13.9 mg/kg bw/day for males and 30.8 mg/kg bw/day for females. While this difference in benchmark dose may indicate that male rats are more sensitive than females, it also can be just a statistical artifact in a rather small sample size, only 10 rats per group. The male rat data set was selected for determining the point of departure for MRL derivation in order to be most conservative. Applying an uncertainty factor of 100 (10 for extrapolation from animals to humans and 10 for human variability) to the BMDL10 of 13.9 mg/kg bw/day yielded an intermediate-duration oral MRL of 0.1 mg/kg bw/day for *m/p*-cresol.

- An MRL of 0.1 mg/kg bw/day has been derived for intermediate-duration oral exposure (15–364 days) to cresols (ATSDR 2008).

Chronic Toxicity (Animal)

The only chronic-duration dietary study with cresols is the NTP (2008) toxicology and carcinogenesis studies in male Fischer-344/N rats and female B6C3F1 mice (NTP 2008). Although the report has not yet been finalized by the NTP, a draft technical report has been reviewed by the NTP Board of Scientific Counselors Technical Reports Review Subcommittee, and a draft abstract, pathology tables, and survival and growth curves are available on the NTP web site. In the study, the male Fischer-344/N rats were fed diets that provided mean time-weighted average (TWA) doses of *m/p*-cresol of approximately 0, 70, 230, or 720 mg/kg bw/day for 2 years. Survival rates were not affected by treatment with *m/p*-cresol. Inspection of the data shows that the most sensitive end point in rats was the nasal respiratory epithelium, as in the shorter-term studies (NTP 1992b). Other less sensitive effects observed in rats included hyperplasia of the transitional epithelium of the renal pelvis, squamous metaplasia in the nasal respiratory epithelium, inflammation of the nose, and eosinophilic foci in the liver. Incidences of respiratory epithelium hyperplasia of minimal to mild severity were 3/50, 17/50, 31/50, and 47/50 in the control, low-, mid-, and high-dose groups, respectively. During the first 13 weeks of the 2-year study, the mean dose in the low-dose group was 123 mg/kg bw/day (calculated from weekly averages provided in the report), the same as in the earlier 13-week study (NTP 1992b), and the incidence of respiratory hyperplasia in this group at termination was 17/50 (34%), almost the same as in the earlier 13-week study, 3/10 (30%). This suggests that, over the range of doses used in these studies, exposure beyond 13 weeks (i.e., duration of exposure) had little or no effect on the incidence or severity of the lesions, indicating that the intermediate-duration MRL is protective of nasal lesions for a 2-year exposure period. This is supported by the fact that fitting the incidence data for nasal respiratory epithelium hyperplasia from the 2-year study to the same BMDS model (Log-Logistic) that provided the BMDL10 used to derive the intermediate-duration oral MRL yields a BMDL10 for chronic exposure to *m/p*-cresol of 13.9017 mg/kg bw/day, essentially the same as the BMDL10 of 13.9381 mg/kg bw/day used to derive the intermediate-duration oral MRL for *m/p*-cresol. Thus, the intermediate-duration oral MRL should be protective of nasal respiratory lesions in rats induced by chronic-duration exposure.

The female B6C3F1 mice were fed diets that provided TWA doses of approximately 0, 100, 300, oral 1,040 mg *m/p*-cresol/kg bw/day for 2 years. Survival rates were comparable among dose groups. Significant treatment-related, non-neoplastic effects occurred in the lung (bronchiole hyperplasia), nose (respiratory epithelium hyperplasia), thyroid gland (follicular degeneration), and liver (eosinophilic foci). In the lung, the incidences of minimal to moderate bronchiole hyperplasia were 0/50, 42/50, 44/49, and 47/50 in the control, low-, mid-, and high-dose groups, respectively. In the nose, the corresponding incidences of minimal to mild respiratory epithelium hyperplasia were 0/50, 0/50, 28/49, and 45/49. In the thyroid, the corresponding incidences of mild follicular degeneration were 7/48, 24/48, 24/49, and 21/50. The corresponding incidences of eosinophilic foci in the liver were 1/50, 0/50, 2/49, and 12/50. Clearly, the thresholds for thyroid follicular degeneration and bronchiole hyperplasia were lower than those for nasal epithelial hyperplasia and liver foci; therefore, the incidence data for the former two lesions were considered for derivation of a chronic-duration oral MRL for *m/p*-cresol. After inspection of the dose response data, the use of a LOAEL/NOAEL approach for MRL derivation was considered to be more appropriate than the use of benchmark dose analysis because of the steep increase in the response rates between the control groups and the first exposure levels. It should be noted that neither bronchiole hyperplasia nor thyroid follicular degeneration were present in female mice in the 13-week study with *m/p*-cresol (NTP 1992b), suggesting that longer periods of exposure were necessary for these lesions to develop. Applying an uncertainty factor of 1,000 (10 for use of a LOAEL, 10 for extrapolation from animals to humans, and 10 for human variability) to the LOAEL of 100 mg/kg bw/day for bronchiole hyperplasia of the lung and follicular degeneration of the thyroid gland in female mice, yields a chronic duration oral MRL of 0.1 mg/kg bw/day for *m/p*-cresol.

- An MRL of 0.1 mg/kg bw/day has been derived for chronic-duration oral exposure (365 days or more) to cresols (summarized in ATSDR 2008).

Reproductive and Developmental Toxicity (Animal)

In addition to the systemic effects observed in the 28-day and 13-week studies (NTP 1992b), exposure to high doses of cresols resulted in reproductive and developmental effects. Mild to moderate uterine atrophy and lengthening of the estrous cycle were generally observed at the highest dose levels tested (>2,000 mg/kg bw/day) for all three isomers. Exposure of mice to 1,682 mg/kg/day *m/p*-cresol caused minor maternal toxicity (reduced body weight gain), decreased number of pups/litter, and increased cumulative days to litter (delay in producing additional F1 offspring). These effects were not observed in mice exposed to 660 mg/kg bw/day *o*-cresol (NTP 1992a).

Two multigeneration reproductive toxicity studies exist in mice dosed with *o*-cresol (NTP 1992a) and a mixture of *m*- and *p*-cresol (NTP 1992c). In the NTP study (1992b, as cited in ATSDR 2008), rats and mice dosed with *p*-cresol or an *m/p*-cresol mixture showed lesions in the nasal respiratory epithelium. The nasal lesions occurred in rats dosed with *p*-cresol for 28 days (≥770 mg/kg bw/day), in rats exposed to *m/p*-cresol for 28 days (≥95 mg/kg bw/day), in mice exposed to *p*-cresol for 28 days (≥163 mg/kg bw/day), in mice exposed to *m/p*-cresol for 28 days (≥ 604mg/kg/day), in rats exposed to *m/p*-cresol for 13 weeks (≥123 mg/kg bw/day), and in mice exposed to *m/p*-cresol for 13 weeks (≥472 mg/kg bw/day). The lesions were located at the most anterior portions of the nasal septum, dorsal arch, and medial aspect of the nasal turbinates. The hyperplasia was characterized by increased number of goblet cells and pseudogland formation due to the infolding of the hyperplastic cells. The hyperplastic areas were associated with single cell necrosis. The intermediate-duration oral gavage studies (EPA 1988b, 1988d, as cited in ATSDR 2008) and two multi-generation reproductive dietary studies in mice (NTP 1992a, 1992c, as cited in ATSDR 2008) did not examine the nasal respiratory epithelium of the animals. Small increases in liver weight were observed in rats and mice at higher doses (≥242 mg/kg bw/day) in both the 28-day and 13-week studies; kidney weight was only increased in rats dosed with ≥861 mg/kg bw/day) *o*-cresol for 28 days. However, the changes in organ weight were not associated with alterations in clinical tests of liver and kidney function or gross and microscopic alterations (NTP 1992b). Decreased weight gain was also observed in rats and mice at relatively high doses (>1,000 mg/kg bw/day).

Mutagenicity

Cresols did not exhibit mutagenicity in tests conducted by the NTP. Each of the individual cresol isomers (*m*-, *o*-, and *p*-) and *m*-/*p*-cresol was tested for mutagenicity in several strains of *S. typhimurium* and in *E. coli* strain WP2, with and without exogenous metabolic activation; results with all individual compounds and the mixture were negative. *o*-Cresol and *m*-/*p*-cresol were evaluated for induction of micronuclei (a biomarker of chromosomal damage) in peripheral blood erythrocytes of male and female mice following 13 weeks of exposure in the diet (NTP, 1992); no increases in the frequencies of micronucleated erythrocytes were seen in male or female mice in either study.

In Vitro Toxicity

Three publications were identified in the literature search update concerning the *in vitro* toxicity of p-cresol. In the first publication, Ying et al., (2011) isolated endothelial progenitor cells (EPCs) from healthy human volunteers and tested for viability and angiogenesis function in vitro following treatment with p-cresol at culture concentrations of 10-80 ug/mL. Treatment with p-cresol inhibited EPC viability in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in a dose-dependent manner and also significantly suppressed angiogenesis capacity as tested in the Matrigel assay.

In the second publication, polymorphonuclear leukocytes (PMNs) were isolated from healthy subjects and treated with p-cresol to investigate the effects of p-cresol on a number of outcomes including apoptosis and cell viability (de Carvalho et al., 2011). Contrary to the effect observed by Ying et al. (2011) in EPCs, the authors did not observe any changes in viability or apoptosis.

In the final study, Chang et al. (2011) tested the effects of p-cresol on a variety of experimental models of platelet aggregation. p-cresol was observed to inhibit arachidonic acid-induced platelet aggregation in a dose-dependent manner in both human and rabbit protein rich plasma. Similar effects however were not observed in U46619-induced models of aggregation. Furthermore, p-cresol was not found to be cytotoxic to platelets. The authors also tested the effects of intravenous administration of p-cresol (250 – 1000 nmole) to mice which resulted in the suppression of *ex vivo* platelet aggregation.

Synopsis for p-cresol

ATSDR and U.S.EPA have evaluated the carcinogenicity data for 4-methylphenol (p-cresol). EPA classifies this chemical as C - possible human carcinogen. The cancer weight of evidence classification is based on all routes of exposure. ATSDR has published a Toxicological Profile for Cresols. Although ATSDR discusses the carcinogenicity data in its Toxicological Profiles, it does not assess cancer potency or perform cancer risk assessments (ITER 2010).

ATSDR, RIVM (the Netherlands), and the U.S.EPA have evaluated the noncancer oral toxicity data for 4-methylphenol (p-cresol). RIVM derived a TDI of 50 ug/kg bw/day (0.05 mg/kg bw/day) based on a marginal LOAEL of 50 mg/kg bw/day for central nervous system (CNS) effects in male rats and application of an uncertainty factor of 1000 (10 each to account for inter- and intraspecies variability and 10 for the lack of a NOAEL). ATSDR derived both an intermediate and chronic oral MRL for p-cresol of 0.1 mg/kg bw/day based on a subchronic study in male rats and a chronic study in female mice (NTP, 2008), respectively. The chronic study data was not available when RIVM evaluated p-cresol. NTP (2008) identified a LOAEL of 100 mg/kg bw/day for bronchiolar hyperplasia and thyroid follicular degeneration observed in female mice. ATSDR applied an uncertainty factor of 1000 (10 each for extrapolation from animals to humans, human variability, and use of LOAEL). The EPA oral reference dose (RfD) for 4-methylphenol has been withdrawn as a result of agency review. A new RfD is in preparation.

Safety Data Summary

Although complete data are not available for some of the components of WMEO, the publically available data that do exist combined with the widely disseminated knowledge concerning the chemistry of isothiocyanates, and the long history of mustard consumption in general provide a sufficient basis for an assessment of the safety of WMEO for the uses proposed herein. The potential toxicity of 4-HBITC, 4-OHBA, 4-OHBCN and p-cresol has been described above. Safe exposure limits (ADI and/or background dietary exposure) for each of the compounds derived from the aforementioned data are as follows:

4-hydroxybenzyl isothiocyanate (4-HBITC)

A broadly based literature search revealed no safety information for 4-HBITC. Some data however were available for the closely related compound 4-BITC. However, the available Absorption Distribution Metabolism Excretion (ADME), short-term toxicity and mutagenicity data provide no basis for estimation of an ADI. However, the background dietary exposure to white mustard seed, based on published information on the concentration of 4-HBITC in white mustard seed and mean daily intake of mustard seed among the U.S. consumers of 1.2 g, allow an estimation of the daily intakes of 4-HBITC from mustard seed range from approximately 14.4 to 40.8 mg (0.24 to 0.68 mg/kg/day for a 60 kg individual). The *per user* 90^{th} percentile estimate of 0.039 mg/kg bw/day from the proposed uses is well below background dietary exposure to 4-HBITC.

4-hydroxybenzyl cyanide (4-OHBCN)

A broadly based literature search revealed only a subcutaneous LDLo in rats of 250 mg/kg. No other safety-related information was available. The *per user* 90th percentile estimate to 4-OHBCN from the proposed new uses of 4-HBITC is 0.0019 mg/kg bw/day is well below the current background dietary intake of 4-OHBCN, which ranges from 2.3 to 6.6 mg (0.038 to 0.11 mg/kg/day for a 60 kg individual).

4-hydroxybenzyl alcohol (4-OHBA)

A broadly based literature search revealed only the results of an *in vitro* Ames assay in S. typhimurium strain TA100 (dose range 50-2000 µg/plate) with 4-HBA, the results of which were negative. No other safety-related information was available.

JECFA (2002) had no safety concerns for 4-OHBA at current levels of intake when used as a flavoring agent. As a flavoring agent, JECFA classified 4-OHBA as a structural class I. These classes of compounds were expected to be hydrolyzed to aromatic aldehydes and simple aliphatic alcohols. Based on the current intake from flavor uses, JECFA stated 4-OHBA had "no safety concern".

Based on published information on the concentration of 4-HBITC in white mustard seed and mean daily intake of mustard seed among the U.S. consumers of 1.2 g, and conservatively assuming that all 4-HBITC formed converts entirely and exclusively to 4-OHBA, the estimated daily intake of 4-OHBA from mustard seed ranges from approximately 10.8 to 30.7 mg (0.18 to 0.51 mg/kg/day for a 60 kg individual). The *per user* 90th percentile estimate of 0.21 mg/kg bw/day from the proposed new uses is within the background dietary exposure. Given the inherent variability in the naturally occurring level of sinalbin in mustard, it is reasonable to assume that the cumulative exposure from the naturally occurring levels of 4-OHBA in the diet and the potential residual levels of 4-OHBA from the proposed use of WMEO would remain within the variable range of natural dietary exposure to 4-OHBA.

p-cresol

The potential toxicity of p-cresol has been studied and a safe level of exposure has been identified by government agencies and regulatory authorities such as the EPA, ATSDR, and RIVM. The available toxicology database consists of acute, subchronic, chronic and genotoxicity studies. A previously published chronic oral RfD for 4-methylphenol has been withdrawn by EPA as a result of agency review and a new RfD is in preparation. RIVM derived a TDI of 50 ug/kg bw/day (0.05 mg/kg bw/day) based on a marginal LOAEL of 50 mg/kg bw/day for CNS effects in a 13-week study rats and application of an uncertainty factor of 1000 (10 each to account for inter- and intraspecies variability and 10 for the lack of a NOAEL). ATSDR derived an intermediate oral MRL of 0.1 mg/kg bw/day based on the results of a subchronic study in male rats and a chronic oral MRL based on results of a chronic (2-year) study in female mice (NTP, 2008) that was not available when RIVM evaluated p-cresol. NTP (2008) identified a LOAEL of 100 mg/kg bw/day for bronchiolar hyperplasia and thyroid follicular degeneration in female mice. Data from the NTP 13-week study were considered adequate for analysis using the benchmark dose approach for the intermediate oral MRL derivation. The male rat data set was selected for determining the point of departure for MRL derivation in order to provide the greatest degree of conservatism. Applying an uncertainty factor of 100 (10 for extrapolation from animals to humans and 10 for human variability) to the BMDL10 of 13.9 mg/kg bw/day yielded an intermediate-duration oral MRL of 0.1 mg/kg bw/day for *m/p*-cresol. ATSDR applied an uncertainty factor of 1000 (10 each for extrapolation from animals to humans, human variability, and use of LOAEL) to the LOAEL of 100 mg/kg bw/day for bronchiole hyperplasia of the lung and follicular degeneration of the thyroid gland in female mice for estimation of the chronic MRL of 0.1 mg/kg bw/day for *m/p*-cresol. The *per user* 90^{th} percentile estimate of 0.0035 mg/kg bw/day from the proposed new uses is well below the intermediate and chronic oral MRLs of 0.1 mg/kg bw/day.

VI. Basis for the GRAS Determination

A. Introduction

The regulatory framework for determining whether a substance can be considered generally recognized as safe (GRAS) for its intended use in accordance with section 201(s) (21 U.S.C. § 321(s)) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. § 301 et. Seq.) ("the Act"), is set forth at 21 CFR 170.30, which states:

> General recognition of safety may be based only on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. The basis of such views may be either (1) scientific procedures or (2) in the case of a substance used in food prior to January 1, 1958, through experience based on common use in food. General recognition of safety requires common knowledge about the substance throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food.
>
> General recognition of safety based upon scientific procedures shall require the same quantity and quality of scientific evidence as is required to obtain approval of a food additive regulation for the ingredient. General recognition of safety through scientific procedures shall ordinarily be based upon published studies, which may be corroborated by unpublished studies and other data information.

These criteria are applied in the analysis below to determine whether the use of WMEO as an antimicrobial in non–carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), ketchup, sauces and gravies in frozen meals, and egg substitutes (e.g., Egg Beaters) is GRAS based upon scientific procedures. All data used in this GRAS determination are publicly available and generally known, and therefore meet the "general recognition" standard for food ingredients under the FD&C Act.

B. Safety Determination

The subject of this GRAS determination is White Mustard Essential Oil (WMEO) proposed for use as an antimicrobial in select foods. Although complete data are not available for some of the components of WMEO, the publically available data that do exist combined with the widely disseminated knowledge concerning the chemistry of isothiocyanates and the long history mustard consumption as a safe food in general, provides a sufficient basis for an assessment of the safety of WMEO for the proposed uses. Establishment of an ADI based solely on available safety data is not feasible for WMEO due to a lack of sufficient data for all of the major hydrolysis products except p-cresol. An acceptable daily intake is conservatively estimated based on available historical consumption data for these compounds in mustards by the U.S. population, and the known and understood safety of consumption at these levels. In the present GRAS determination, the use of historical consumption data to evaluate safety has two distinct advantages. First, historical dietary consumption data in the human population better represent WMEO consumption than laboratory tests conducted in non-human species, and second, dietary consumption by the human population better reflects the safety of the unique mixture of compounds and hydrolysis products found in WMEO than safety data on the individual components described above.

Safe exposure limits (ADI and/or background dietary exposure) for each of the compounds derived from the aforementioned data are as follows:

4-hydroxybenzyl isothiocyanate (4-HBITC): The background dietary exposure to white mustard seed, based on published information on the concentration of 4-HBITC in white mustard seed and mean daily intake of mustard seed among the U.S. consumers of 1.2 g, allow an estimation of the daily intake of 4-HBITC of approximately 14.4 to 40.8 mg (0.24 to 0.68 mg/kg/day for a 60 kg individual).

The *per user* 90th percentile estimate of 0.039 mg/kg bw/day from the proposed use of WMEO as an antimicrobial in non–carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), ketchup, sauces and gravies in frozen meals, and egg substitutes (e.g., Egg Beaters) is well below background dietary exposure to 4-HBITC.

4-hydroxybenzyl cyanide: Published information available describing the maximum amount of 4-OHBCN generated from the hydrolysis of the major WMEO component sinalbin and the mean daily intake of mustard seed among the U.S. consumers of 1.2 g, allow an estimation of the daily intake of 4-OHBCN from mustard seed of approximately 2.3 to 6.6 mg (0.038 to 0.11 mg/kg/day for a 60 kg individual).

The *per user* 90th percentile estimate to 4-OHBCN from the proposed new uses of as an antimicrobial in non–carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), ketchup, sauces and gravies in frozen meals, and egg substitutes (e.g., Egg Beaters) is 0.0019 mg/kg bw/day which is well below background dietary exposure to 4-OHBCN.

4-hydroxybenzyl alcohol: Based on published information on the concentration of 4-HBITC in white mustard seed and mean daily intake of mustard seed among the U.S. consumers of 1.2 g, and the conservative assumption that up to 100% of the 4-HBITC formed from sinalbin may in turn form 4-OHBA, the estimated daily intake of 4-OHBA from mustard seed ranges from approximately 10.8 to 30.7 mg (0.18 to 0.51 mg/kg bw/day for a 60 kg individual).

The *per user* 90th percentile estimate of 0.21 mg/kg bw/day from the proposed use of WMEO as an antimicrobial in non–carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), ketchup, sauces and gravies in frozen meals, and egg substitutes (e.g., Egg Beaters) is within and at the low end of the background dietary exposure to 4-OHBA. Given the inherent variability in the naturally occurring level of sinalbin in mustard, it is reasonable to suggest that the cumulative exposure from the naturally occurring levels of 4-OHBA in the diet and the potential residual levels of 4-OHBA from the proposed use of WMEO in select foods would remain within the variable range of natural dietary exposure to 4-OHBA.

p-cresol: The potential toxicity of p-cresol has been studied and a safe level of exposure has been identified by government agencies and regulatory authorities such as the EPA, ATSDR, and RIVM. The available toxicology database consists of acute, subchronic, chronic and genotoxicity studies. A previously published chronic oral RfD for 4-methylphenol has been withdrawn by EPA as a result of agency review and a new RfD is in preparation. RIVM derived a TDI of 50 µg/kg bw/day (0.05 mg/kg bw/day) based on a marginal LOAEL of 50 mg/kg bw/day for CNS effects in a 13-week rat study and application of an uncertainty factor of 1000 (10 each to account for inter- and intraspecies variability and 10 for the lack of a NOAEL). ATSDR derived an intermediate oral MRL of 0.1 mg/kg bw/day based on the results of a subchronic study in male rats and a chronic oral MRL based on results of a chronic (2-year) study in female mice (NTP, 2008) that was not available when RIVM evaluated p-cresol. NTP (2008) identified a LOAEL of 100 mg/kg-day for bronchiolar hyperplasia and thyroid follicular degeneration in female mice. Data from the NTP 13-week study were considered adequate for analysis using the benchmark dose approach for the intermediate oral MRL derivation. The male rat data set was selected for determining the point of departure for MRL derivation in order to provide the greatest degree of conservatism. Applying an uncertainty factor of 100 (10 for extrapolation from animals to humans and 10 for human variability) to the BMDL10 of 13.9 mg/kg bw/day yielded an intermediate-duration oral MRL of 0.1 mg/kg bw/day for *m/p*-cresol. ATSDR applied an uncertainty factor of 1000 (10 each for extrapolation from animals to humans, human variability, and use of LOAEL) to the LOAEL of 100 mg/kg bw/day for bronchiole hyperplasia of the lung and follicular degeneration of the thyroid gland in female mice for estimation of the chronic MRL of 0.1 mg/kg bw/day for *m/p*-cresol.

The *per user* 90th percentile estimate of 0.0035 mg/kg bw/day from the proposed use of WMEO as an antimicrobial in non–carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), ketchup, sauces and gravies in frozen meals, and egg substitutes (e.g., Egg Beaters) is well below the intermediate and chronic oral MRLs of 0.1 mg/kg bw/day.

Conclusion

The proposed use of WMEO as an antimicrobial in non–carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), ketchup, sauces and gravies in frozen meals, and egg substitutes (e.g., Egg Beaters) provides 90th percentile estimated daily intakes (EDIs) for 4-HBITC, 4-OHBA, 4-HBCN, and p-cresol for the U.S. population that are either below an established regulatory benchmark (e.g., p-cresol) or at or below the range of background dietary exposure to the compounds from consumption of mustard. Therefore, the proposed use of WMEO is safe with the meaning of the FD&C Act, i.e., it meets the standard of reasonable certainty of no harm.

C. General Recognition of the Safety of WMEO

The intended use of WMEO as an antimicrobial in non–carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), ketchup, sauces and gravies in frozen meals, and egg substitutes (e.g., Egg Beaters) has been determined to be safe through scientific procedures as set forth in 21 CFR§170.30(b), thus satisfying the so-called "technical" element of the GRAS determination. Because this safety evaluation was based on generally available and widely accepted data and information, it also satisfies the so-called "common knowledge" element of a GRAS determination. Determination of the safety and GRAS status of WMEO for addition to foods under its intended conditions of use has been made through the deliberations of an Expert Panel comprised of Stanley M. Tarka, Jr., Ph.D., Francis F. Busta, Ph.D , and Eric Wilhemsen, Ph.D. These individuals are qualified by scientific training and experience to evaluate the safety of substances intended to be added to food. They have critically reviewed and evaluated the publicly available information summarized in this document and have individually and collectively concluded that WMEO, produced consistent with Good Manufacturing Practice and meeting the specifications described herein, is safe under its intended conditions of use. The Panel further unanimously concludes that these uses of WMEO are GRAS based on scientific procedures, and that other experts qualified to assess the safety of foods and food ingredients would concur with these conclusions. The Panel's GRAS opinion is included as Exhibit I to this document.

ConAgra Foods Inc. is not aware of information that would be inconsistent with a finding that the proposed use of WMEO as an antimicrobial in non–carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), ketchup, sauces and gravies in frozen meals, and egg substitutes (e.g., Egg Beaters) meeting appropriate specifications and used according to Good Manufacturing Practice, is GRAS. A recent search of the scientific literature revealed no potential adverse health concerns.

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Appendices

Appendices

Appendix A

Appendix A. ConAgra Laboratory Reports

Documentation for safety evaluation of white mustard (*Sinapis alba*) essential oil as a natural food preservative.

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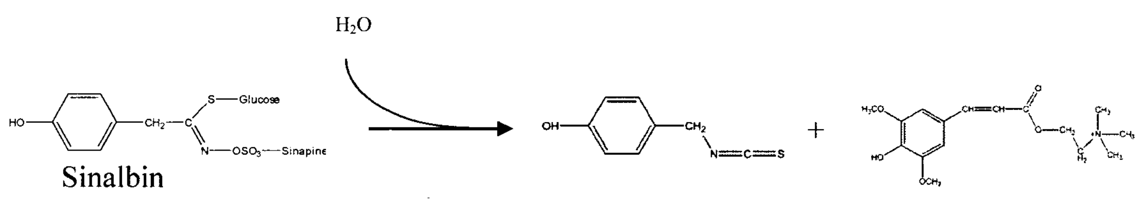
Introduction:

Of the three varieties of mustard seeds black (*Brassica nigra*), oriental (*Brassica juncea*) and white or yellow (*Sinapis alba* or *Brassica hirta*) cultivated today only white mustard contains the glucosinolate sinalbin almost exclusively. When white mustard seeds are ground and moistened, sinalbin is hydrolyzed to 4-hydroxybenzyl isothiocyanate (4HBITC), a non-volatile, sharp tasting oily compound. This reaction is catalyzed by the endogenous enzyme myrosinase (E.C. 3.2.3.1 thioglucoside glucohydrolase) and myrosinase is further activated by low concentrations of ascorbic acid.



4HBITC has wide spectrum antimicrobial properties and is cidal to bacteria, yeast and fungi at relatively low concentrations. It is also unstable in aqueous systems hydrolyzing to 4-hydroxybenzyl alcohol primarily. The stability of 4HBITC in aqueous systems is pH dependant and is more stable in acids (few days) and less stable with increasing pH, instantaneously decomposing at alkaline pHs'.

To prepare 4HBITC commercially as part of white mustard essential oil (WMEO), mustard seeds are first cold pressed to remove most of the fixed oil and the partially defatted mustard press cake is moistened in the presence of ethyl acetate and the activator ascorbic acid. After the reaction period, the ethyl acetate extract containing 4HBITC and the fixed oil in the starting partially defatted mustard press cake, is removed by centrifugation. Low temperature evaporation of ethyl acetate under reduced pressure yields white mustard essential oil (WMEO) containing 4HBITC and some of the fixed oil from the starting partially defatted mustard press cake.

In order to stabilize 4HBITC in the WMEO and also provide a means of easy dispensing, WMEO is mixed with maltodextrin (Ekanayake, A. et al. 2006) to provide an easily dispensable powdery material. It can also be diluted with a vegetable oil, dry powdered sugar or salt, for this same purpose.

This document gives the details pertaining to 1a) Large scale manufacture of WMEO starting from white mustard seed. 1b) Purification of the active compound 4HBITC for analytical purposes. 2) Mass spectral analysis of WMEO compared to the cold pressed mustard oil. 3) Hydrolysis studies at pH 3.5, 6.0 and 9.0 buffers and roasted red bell pepper puree. 4) Mass balance of 4-HBITC hydrolysis products.

Summary of findings:

White mustard essential oil (WMEO) can be made in large quantity by following the sequence of unit operations given below.
1) Cold pressing (optional) yellow or white mustard seed. 2) Grinding the press cake or seed to a fine powder. 3) Moistening the ground mustard; activating the myrosinase enzyme and simultaneously extracting the residual oil and 4-hydroxybenzyl isothiocyanate (4HBITC) by solvent extraction. 4) Separating the ethyl acetate solution containing 4HBITC and residual mustard oil from the moistened mustard using centrifugation. 5) Removing the solvent ethyl acetate from the solution of Step 4, by low temperature evaporation. 6) Freezing the resulting WMEO or mixing with maltodextrin, vegetable oils, powdered sugar or salt to obtain a more stable and easily dispensable food ingredient.
A pure sample of 4HBITC was prepared using WMEO and using solvent fractionation to remove the mustard oil and enrich 4HBITC before purification by chromatographic techniques.
Mass spectrometric analysis showed that the triglyceride fraction of the WMEO was identical to that of the cold pressed mustard oil and that the additional compounds found in WMEO are primarily 4HBITC and other compounds of Brassica origin.
Over 15 compound formulae have been determined by combined ultra-high-pressure liquid chromatography (uHPLC) - electrospray ionization - time-of-flight - mass spectrometry (uHPLC-ESI-Tof-MS) in the analysis of a dissolved "processed-pressed oil" extract (= WMEO) of white mustard (Brassicacae family) seeds. By comparing the formulae to those already reported in the phytochemical literature for this family, it is hypothesized that the compounds are naturally occurring and may react by known chemistry, adding a p-OH-benzyl moiety (derived from naturally occurring p-OH-benzyl isothiocyanate). Mass errors were generally less than ± 2 ppm for a proposed formula, which also always produced the top-ranked fit of the observed isotope patterns in the list of candidate formulae within 10 ppm of the observed m/z value. The use of uHPLC-ESI-Tof-MS under conditions of 100V potential on the entrance cone generated fragment ions consistent with expected sub-structural units.
Newly developed HPLC methods were used to track the hydrolytic fate of 4HBITC and its degradation compounds in the series of hydrolysis experiments. These experiments were carried out by adding the WMEO in maltodextrin preparation to pH 3.5, 6.0 and 9.0 buffers which showed that the active component 4HBITC was unstable in aqueous solutions. The relative stability at pH 3.5 was much higher with measurable amounts surviving for a few days, compared to pH 6.0 where it was below detection levels in 48 hours. Hydrolysis at pH 9.0 was instantaneous with no measurable amounts after 2 hours.
The hydrolysis reactions were all first order with a tendency to shift towards second order reactions with increasing pH. Hydrolysis in an actual vegetable puree, roasted red bell pepper – pH 4.97, followed the above trend.
The non-Sulfur containing hydrolysis products were 4-hydroxybenzyl alcohol (4OHBA), 4-hydroxybenzyl cyanide (4OHBCN) and p-cresol. Literature indicates the almost quantitative formation of ionic thio-cyanate (SCN^-) as the sulfur containing moiety from the hydrolysis reaction of 4HBITC.

Production of white mustard essential oil and preparation of 4HBITC analytical standard:

This report describes the industrial scale preparation of white mustard essential oil (WMEO) that was used for the qualitative analysis and hydrolysis experiments and the purification of 4HBITC from a lab prepared sample of WMEO.
The industrial scale preparation of WMEO also resulted in the production of cold pressed mustard oil which was used as the sample for comparing the triglyceride profile of WMEO to ensure that no change had taken place during WMEO processing.
The 4HBITC component of WMEO responsible for its antimicrobial activity is not currently available commercially and has to be custom synthesized. Due to its instability particularly during the subsequent clean up steps following synthesis, we have not been able to procure a pure synthetic sample commercially. We decided to purify 4HBITC from a lab prepared WMEO sample that had about 15% 4HBITC as assayed by normal phase HPLC analysis. This pure sample was used in all of our current studies.

White mustard essential oil production:

Materials & Methods:

Cold pressing white mustard seed:
White mustard seed (800 kg) was cold pressed using a Rosedowns Mini 200 screw press unit. The resulting press cake exited the press at about 75-78°C and was immediately cooled to room temperature by hammer milling. The oil content of the press cake was 17%. The cold pressed oil was centrifuged at 3700 rpm for 15 min. at room temperature to remove the residual plant debris and stored at -25°C until analyzed as part of the qualitative analysis of the mustard oils.

WMEO generation & solvent extraction:
Hammer milled white mustard press cake (340 kg) from above unit operation was introduced into a ribbon blender and ascorbic acid (78 g) added to it while blending to ensure uniform mixing. Room temperature water (102 kg) was added in small proportions to ensure uniform wetting of the press cake. After mixing for about 10-15 min., the moistened and myrosinase enzyme activated press cake was transferred to a stirred solvent tank for extracting with ethyl acetate (626 kg). The moistened, activated press cake and ethyl acetate slurry was stirred within the closed solvent tank for about 4 hours at room temperature, to ensure generation and transfer of the 4-hydroxy benzyl isothiocyanate from the moist white mustard press cake into the ethyl acetate.

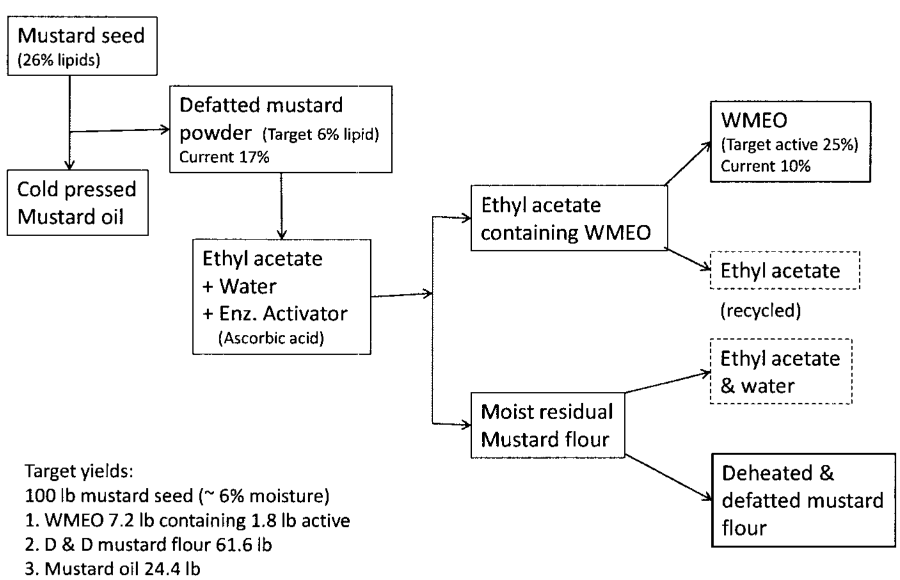
Solvent separation & evaporation:
At the end of the reaction period the slurry of moist white mustard press cake in ethyl acetate was pumped into a decanter centrifuge to separate the ethyl acetate from the moist white mustard press cake. The ethyl acetate layer was collected in a static solvent tank while the wet white mustard press cake with some residual ethyl acetate was directly transferred into a vacuum assisted belt dryer. Here the vacuum conditions allowed the moist ethyl acetate to leave the mustard press cake and get condensed in an external condenser. The ethyl acetate top layer was separated out and stored for re-use while the water layer was discharged. The mustard press

cake continued to travel on a continuous belt towards the bottom of the dryer where the dried, solvent free mustard press cake exited via a screw conveyor. This deheated and defatted mustard flour was analyzed for its gross composition and its amino acid composition. The ethyl acetate layer containing the WMEO was pumped into a rising film evaporator kept under about 56 cm Hg vacuum to remove the ethyl acetate from the white mustard essential oil (WMEO). The evaporation step resulted in about 65.5 kg of WMEO. Small analytical samples (10-15 g) were removed and kept frozen at -25°C until analysis.

Maltodextrin mixing to prepare ingredient:
Maltodextrin (549 kg, 15 DE, Grain Products Corp., Muscatine, IA) was introduced into a paddle mixer (LittlefordDay Model FKM 600-D, Florence, KY) and mixing begun. WMEO (61 kg) was dripped into the fluidized maltodextrin within the mixer and the mixing continued for about 20 additional minutes after addition of the WMEO was complete to ensure homogeneity of the final product. The WMEO+MD ingredient was bagged in drums lined with plastic bags and stored refrigerated until use. Samples of this ingredient were also removed for analytical verification of the 4HBITC concentration.

WMEO analysis:
WMEO was analyzed both qualitatively and quantitatively. Qualitative analysis was done to determine that the triglyceride composition of the cold pressed mustard oil and of the solvent extracted WMEO were essentially similar. The nature of the N and S containing compounds present in the WMEO was also determined. Quantitative analysis was carried out to determine the exact concentration of 4HBITC in WMEO.

WMEO Extraction – flow chart



4-hydroxybenzyl isothiocyanate purification:

Mechanically defatted mustard seed, containing active myrosinase enzyme and thus capable of producing 4HBITC on wetting with water, was obtained from Botanic Oil Innovations Inc., Spooner, WI. Two kg of the above powder was placed in a large plastic pail and 0.176 g ascorbic acid added to it with stirring. After mixing the powders for about 1 minute with an electric hand held mixer, 600 mL distilled water was added in small portions with continuous mixing to ensure even dispersion of the water. After mixing for about 10-15 minutes a physically uniform, moistened mustard powder resulted with the consistency of dry dough. This was placed in a 10 L glass reactor equipped with a top drive stirrer and 4 L of ethyl acetate added to the reactor. Stirring was begun with the ethyl acetate addition and continued for a further 4 hours at room temperature (21°C).

At the end of this period, the slurry was centrifuged for 15 minutes at 3700 rpm to separate the moistened white mustard seed pellet. The supernatant ethyl acetate (3.5 L) solution of the fat soluble materials from the white mustard seeds was dried over anhydrous sodium sulfate for 4-6 hours and the solvent removed under reduced pressure to yield about 380 g of white mustard essential oil (WMEO), a red colored material.

This oil contained about 14% 4HBITC based on peak area measurements of the HPLC chromatogram trace. It was stored frozen at -25°C.

About 60 g of the WMEO was gently contacted with hexane repeatedly (4x80 mL) and the oil layer and hexane layer separated by decanting. The pooled hexane extract was extracted with methanol (4x80 mL). The methanol layer was back extracted with hexane and the methanol layer and the oil layer from the two extractions pooled and the solvent removed under reduced pressure to yield about 12.8 g 4HBITC. For further purification 5 g of the above material was dissolved in 14 mL dichloromethane to which 6 mL of hexane was added. This solution was used as the feed material for the Biotage Chromatography system. A KP-Sil (32-63 cm, 60 A) column conditioned by washing with 4 L of ethyl acetate followed by 70% dichloromethane in hexane was loaded with the feed solution described above and eluted with 5 L of 80% dichloromethane in hexane followed by 2L of 90% dichloromethane in hexane at a flow rate of 320-350 mL/min. The eluate was monitored by thin layer chromatography (TLC). Fractions of 500 to 900 mL were collected and the following fractions were pooled based on their TLC. Fractions 4 to 9, Fractions 2 & 3 and Fractions 10 to 12 were pooled separately and evaporated to dryness under vacuum not exceeding 30°C. This procedure was repeated two more times to further purify the 4HBITC to homogeneity. The purity was checked by chromatography using the normal phase method described in the hydrolysis section of the report. Proton and ^{13}C nmr was obtained to confirm purity of sample. The final standard material was individually packaged in amber colored glass vials that were tared and flushed with nitrogen with the final weight of each vials recorded for ease of handling the standards. The vials were stored desiccated at -25°C until use.

Results & Discussion:

As seen in Figure 1, the residual lipid content in the cold pressed mustard seed controls the concentration of 4HBITC in WMEO provided that the conditions for generating 4HBITC from the myrosinase catalyzed system have been largely optimized. This is because ethyl acetate is a good solvent for lipophilic materials including all lipids and 4HBITC. If mustard seed, without any pressing, is used as the starting material the concentration of 4HBITC in WMEO is about 4-

5% and in theory if mustard seed is completely defatted, by the use of hexane for example, the concentration of 4HBITC will be in the 90% range. However this latter mode of defatting is probably not economical from an industrial point of view unless high value added uses are found for the residual high protein mustard flour, which will offset some of the additional solvent use costs. In the current extraction the residual lipid content in the cold pressed mustard seed was about 17% giving a 4HBITC concentration of about 8-10% in the WMEO. The HPLC chromatogram and proton and C^{13} nmr spectra (Figure 2) for the 4HBITC isolated from WMEO show it to be about 93% pure and sufficient for use to quantify 4HBITC in extracts as well as the hydrolysis experiments. Spectral assignments are described in Pedras & Smith (1997).

REFERENCE:

Pedras, S.M. and Smith, K.C. 1997. Sinalexin, A Phytoalexin from white mustard elicited by destruxin B and alternaria brassicae. Phytochemistry; 46(5) 833-837.

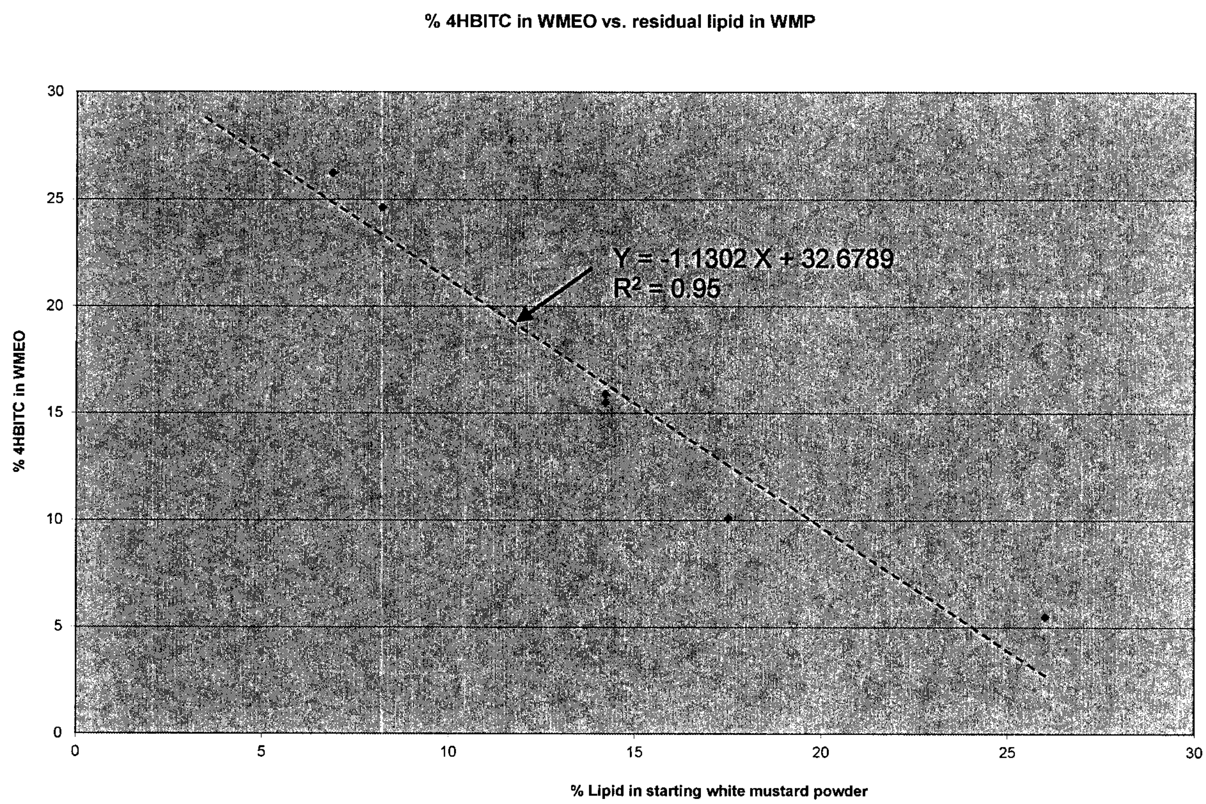


Figure 1. Concentration of 4HBITC in WMEO as a function of the residual lipid concentration in the starting white mustard powder.

Figure 2. HPLC chromatogram, proton and C^{13} nmr data for 4HBITC purified from WMEO. NMR peak assignments are as per Pedras & Smith (1997).

D-7000 HPLC System Manager Report

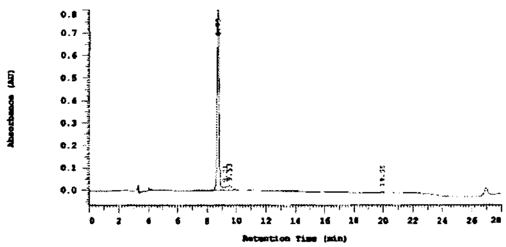
Analyzed: 09/11/09 12:30 PM

Reported: 09/11/09 02:46 PM
Processed: 09/11/09 02:46 PM

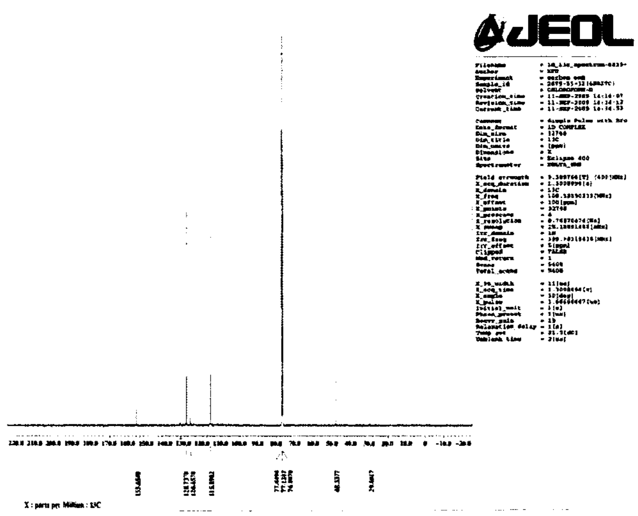
Data Path: D:\P & G\DATA\0007\
Processing Method: 4HBITC
System(acquisition): 1501
Application: 4HBITC
Sample Name: 2675-55-32 in DCM
Injection from this vial: 1 of 1
Sample Description:

Series:0007
Vial Number: 4
Vial Type: UNK
Volume: 10.0 ul

Chrom Type: Fixed WL Chromatogram, 277 nm



No.	RT	Area	Height	BC	Conc 1 mg/mL	Area %
1	8.73	3677275	425851	MC	0	92.874
2	9.21	94620	5753	MC	0	2.390
3	9.53	176733	9154	MC	0	4.464
4	19.95	10780	1512	BB	0	0.272
		3959408	442270		0	100.000



Qualitative analysis of white mustard essential oil:

1) Solubility Properties - the Mustard oils ("native" cold pressed seed oil versus seed oil processed to enhance p-OH benzylisothiocyanate content) were found to be soluble at 1 mg/mL in 50/50 acetonitrile (ACN) / isopropanol (IPA) as judged by solution clarity and lack of any fine particulates. Weakening the solvent further (lower IPA, or water addition) causes partial solubility conditions (cloudiness). This is largely due to the fact that the sample is approximately 90% triglycerides. However, it was found that 2-3 microliter of this solution could be injected at initial HPLC solvent conditions as weak as 10% ACN in 90% water, without deleterious effects on HPLC analysis (e.g., sample precipitation, high pressure build up or column plugging).

2) Combined HPLC/MS: Each sample was subject to two analyses by separate HPLC methods using the Waters Aquity uHPLC interfaced to the Waters Premier time-of-flight mass spectrometer (TOF-MS). The total flow was directed through a dual-wavelength UV detector connected in serial fashion to the mass spectrometer. The first HPLC analysis focused on triglyceride (TG) fingerprinting, using our standard approach detailed below. The second HPLC method focused on separating the more polar components of the sample

HPLC METHOD 1 - An xBridge-RP18 column was used (2.1 mm i.d. x 50 mm length, 2.5 μm particles, p/n 186003092, Waters).

A gradient where A= 80/18/2 acetonitrile (ACN) / Isopropanol (IPA) / 50 mM ammonium formate and B = 50 /48 /2 ACN / IPA / 50 mM aqueous ammonium formate was set up. (Note that the acetonitrile is carefully added to the stock 50 mM ammoniun formate solution in small portions, with swirling, when making up the HPLC solvent). The final concentration of the buffer is 1 mM (i.e., 2 mL of stock 50 mM solution in 100 mL total volume, a 50x dilution).

TIME (Min)	%A	%B	flow (mL/min)
0.00	100	0	0.25
7.00	100	0	0.25
25.00	10	90	0.25
28.00	10	90	0.25
30.00	90	10	0.25
31.00	100	0	0.25
35.00	100	0	0.25

METHOD 2 - The column was switched to a uHPLC HSS T3 functionalized C18, for polar compound retention (2.1 mm i.d. x 50 mm length, 1.8 μm particle size, p/n 186004055, Waters) which separates compounds like cyclobrassinins and isothiocyanates. In this latter analysis, the TG's are simply trapped at the head of the column and remain there through the conclusion of the analysis of the non-TG fraction, and are then removed using an IPA/ACN wash. The column is re-equilibrated to weak conditions prior to the next analysis.

A1 = 88/2/10 distilled water / 50 mM Aq. ammonium formate / ACN; **B1** = 2/98 50 mM ammonium formate / ACN; **A2** = 50/48/2 ACN / IPA / 50 mM ammonium formate.

Time (min)	% A1	%B1	%A2	Flow (mL/min)
0.00	90	10	0	0.25
20.00	5	95	0	0.25
24.90	5	95	0	0.25
25.00	5	95	0	0.25
0.00 start TG rinse	0	100	0	0.25
20.00	0	0	100	0.25
22.00	0	100	0	0.25
25.00	0	100	0	0.25
40.00	90	10	0	0.25

3) Mass Spectrometry: Ionization of the HPLC effluent is performed via electrospray ionization (ESI, typically 3.5 kV in positive ion and 2 kV in negative ion modes). The cone voltage was 30-32 V typically. The (+) ESI runs and (-)ESI runs were performed separately. A typical scan range is 65μ-1250μ with a cycle time of 0.5 seconds. Calibration was carried per the manufacturer's instructions using a fresh solution of sodium formate (aqueous, 100 μg/mL infused at 10 μL/min). In the case of separate structural-probing experiments (non-TG fraction), the electrospray cone voltage was set to 100 volts, to induce fragment ion formation in the source region.

4) Results and Discussion:

Triglyceride Components:
The use of 1 mM ammonium acetate in the HPLC solvents facilitates ionization of triglycerides as $[M+NH_4]^+$ adduct ions. The observed mass accuracies were on the order of 1-5 ppm. Specific triglyceride elution profiles (for example all C_{57} species with a total of three side-chain double bonds) were obtained by "reconstructing" the HPLC-MS data into accurate mass ion chromatograms (so-called RIC's). In this way, specific TG peak areas were integrated for a particular member of the class, and comparisons between all classes are summarized in the bar charts.

The summary bar charts for the triglycerides are shown in Figure 3. They are based on a single injection for each sample. The analytical method has long been used in other Procter & Gamble projects aimed at TG analysis in oils and we are familiar with how to handle such samples in SFC and HPLC applications. The similarity of the two sets of bar charts is remarkable and the slight variations in some bars are well within run-to-run variations that one might expect.

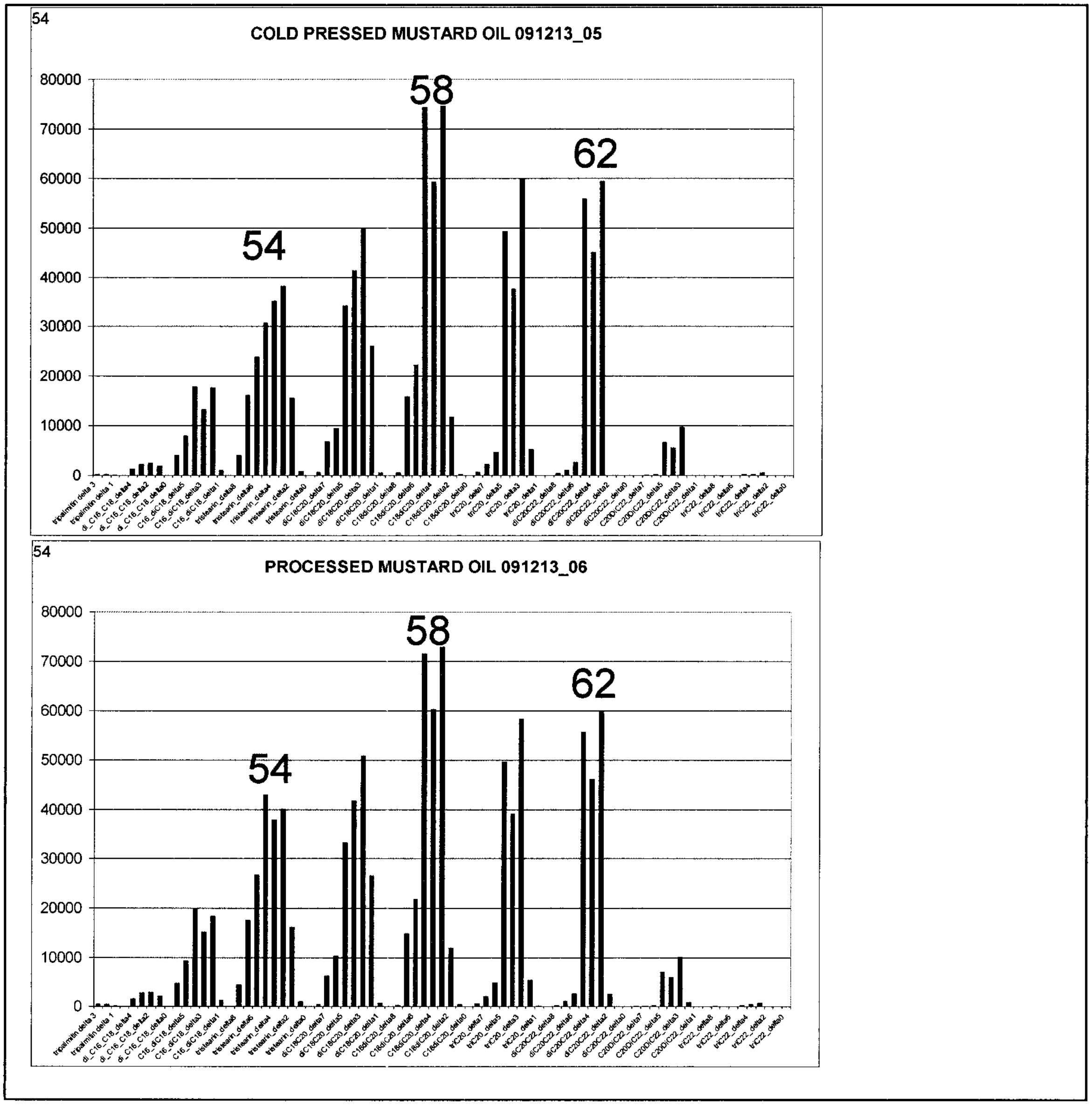


Figure 3. Summary triglyceride distribution bar charts for cold pressed (upper) and processed (lower) mustard oils. The numbers above bars refer to total side chain carbon content. Within a group, unsaturation content runs from higher to lower, left to right. The group numbers advance by two-carbons due to the biosynthetic path of fatty acids in plants.

Non-Triglyceride Components:
The seeds of the White mustard plant (*Sinapis alba,* family Brassicacae) have been reported as a source of natural anti-microbial / preservative activity, putatively associated with the presence of p-hydroxybenzyl isothiocyanate [1], or PHBITC. If the natural oil is processed to enhance PHBITC, then it is pertinent to examine how other naturally occurring compounds in this plant family, e.g., brassinins, are enhanced or perhaps react, during processing.

Sinalbin (Figure 4, a naturally occurring material) is a salt composed of an organic cation (I) and an organic anion (II). It has been reported to decompose to a family of compounds under the action of the hydrolytic enzyme myrosinase [2,3]. Some of these structures (III-VIII) and their formulae are shown in Figure 5. It has been reported that after the glycolytic cleavage catalyzed by myrosinase, that nucleophiles (e.g., ascorbate anion) will substitute at the benzylic position of VI (PHBITC) to make various p-hydroxybenzyl compounds [4]. Phytoalexins including the brassinins and sinalexins [5] may also be naturally present in plants of the Brassicacae family (Figure 6, IX-XIV). Products that may derive generally from the glucosinolate function have also been reviewed [6]. Finally, another class of compounds, the flavonol glycosides ($C_xH_yO_z$ compositions) has also been reported in Brassica and Sinapis. [7].

The chemical formulae, double-bond equivalents (DBE) and N/O/S count of atoms in compounds reported in the phytochemical literature of the Brassicacae family are summarized in Table 1. These formulae are a useful point of reference for similar or related compound formulae discovered in this work.

A comparison of a blank injection chromatogram versus native "cold pressed" mustard seed oil processed oil (mild extraction) is shown in Figure 7 (bottom and middle traces). The native oil trace is virtually identical to the blank injection and is reproducible. The processed oil sample (Figure 7, top trace) is clearly different, showing components that have either been formed in processing or enhanced in concentration by the solvent extraction.

The formulae of the compounds associated with the few HPLC peaks observed in the blank and native oil traces match known surfactants with low degrees of unsaturation and relatively high mass defects, due to their relatively high hydrogen (mass = 1.007825) content. These compounds are "environmental" contaminants often observed in this type of analysis because of their relatively high response factors in electrospray ionization.

The processed oil sample is clearly different, showing components that have either been formed in processing or enhanced in concentration by the extraction. These compounds are more polar and are not expected to be oil-soluble and are likely left behind in the matrix "cake" after cold pressing the seeds. Thus they may be subsequently, mildly extracted using the relatively polar solvent, ethyl acetate. The chemical treatment is very mild (e.g., no pH extremes) and similar to processes already reported in the literature. Thus, chemical alterations of natural materials seem unlikely.

However, it is known that PHBITC itself is "reactive". For instance, if PHBITC is present at ten weight percent, pseudo-first-order kinetics are likely for subsequent bimolecular reactions with other, relatively lower concentration materials, such that:

$$-d(A)/dT = k_{app}(A) \text{ where } (A) = \text{the concentration of PHBITC.}$$

Since the isothiocyanate anion is a good leaving group, essentially PHBITC is a source of the p-OH-benzyl electrophile. Merely enhancing the PHBITC concentration will increase initial rates of substitution reactions. When determining new structures, this general reaction may be an important consideration.

General Observations: A summary of the compounds found by LC/MS, ordered by increasing retention time in HPLC, is shown in Table 2 and includes their peak designations (based on historical work in this laboratory), the formulae of the neutral forms, M^0, their accurate atomic masses, respective N/O/S atom counts, and brief comments.

Note that both positive and negative ESI work was performed. Compounds designated by a letter (A-F) were only observed in the positive ion mode. Compounds "4", "8" and "10" were only observed in the negative ion mode. All other compounds were observed in both modes. Formulae of M^0 were often confirmed by the measurement of more than one mass spectral peak in a spectrum; for example, MH^+, MNH_4^+, MNa^+, MK^+ in positive ion mode or $[M-H]^-$, and $[M-H]^- \cdot HCOOH$ in the negative ion mode. Most proposed formulae fell within 2 ppm of theoretical masses. When all possible formulae within 10 ppm of the observed mass were considered, the formulae reported in Table 2 was always the top ranked formulae in the candidate list, by natural-isotopic pattern fit to the observed data. Finally, the only standard available for comparison was PHBITC.

Specific HPLC Peak Identities - Summary comments are listed in Table 2. Some other additional comments are listed below for specific components, ordered by retention time.

"Q" - The data for "Q" suggest this compound is sinapine, the organic cation of Sinalbin (I, Figure 4). The counter anion II, Figure 4, was not found in negative ion work. By increasing the entrance cone voltage, "Q" was found to lose trimethyl amine (TMA) and (TMA+ ethylene oxide), fully consistent with the proposed structure. These "front-end-fragmentation" experiments will be simply referred to as "high cone" within.

"1" - This relatively weak component had a formula that stoichiometrically is equivalent to three p-OH-benzyl moieties with one nitrogen atom. The exact arrangement of the groups could not be determined by high cone experiments. Nucleophilic substitution on PHBITC with displacement of isothiocyanate anion is well known. The decomposition product p-OH-benzyl amine (V) has been reported [2] so perhaps this compound is simply further substitution.

"2" - This component had a formula very similar to IV, a reported decomposition product. It is proposed that C=S in IV is converted to C=O in "2" by hydrolysis. Hi-cone experiments produced several fragments associated with the p-OH-benzyl substructure, for example m/z 107, $C_7H_7O^+$ by accurate mass measurement. Unfortunately, this pathway dominates the fragmentation of many of the compounds reported within, so that other linkages like, the NH-CO-S proposed here, are not observed and therefore stand as logical but untested hypotheses.

"A" - Stoichiometrically, this peak is equivalent to four p-OH-benzyl moieties on nitrogen, which is consistent with the chemistry proposed for formation of compound 1.

"B" - Brassinin (XII, Figure 6) is naturally occurring and 7-2/1/2 by DBE-N/O/S count designation. The compound "B" fits 11-2/2/2which is consistent with electrophilic substitution of p-OH-benzyl (add 4-0/1/0 to DBE-N/O/S count). A simple electron density analysis of the heterocyclic indole predicts several activated ring sites for reactive addition of p-OH-benzyl.

Alternatively, the indole ring can be viewed as "pushing electrons" onto PHBITC and nucleophilically displacing the thiocyanate anion, a weak base and good leaving group. Consistent with the idea of several activated positions on the indole is the reconstructed ion chromatogram of m/z 295.0569 at 25 ppm tolerance, which show many HPLC peaks (isomers of this formula; see within).

"C" , "D", "7" - Compound "7" is the dominant peak in the HPLC trace based on positive ion ESI and shows several isomers including "C' and "D" and minor peaks designated as i-7 (Figure 8). The formula of this group is consistent with plant metabolism though it has not been previously reported to our knowledge. The formula corresponds to N-methoxy-cyclobrassinin, IX (Figure 6) substituted with an *additional* methoxy group (oxidation and O-methylation). Oxidation could occur at many different sites on the molecule. There is evidence for -OCH_3 substitution on the indole ring, as under hi-cone conditions, the compound forms m/z 107 as already described.

"3", "4" - Compound "3" corresponds stoichiometrically to addition of p-hydroxybenzyl moiety to the know decomposition product IV. The exact bond linkages are unknown. Sulfur release has been reported form sinalbin under mildly acid conditions (pH=4) and the formula of compound 4 corresponds stoichiometrically to addition of [2 x p-hydroxybenzyl] moiety to sulfur. The corresponding disulfide form of this structure has been reported (III, Figure 5)

PHBITC, "5" - The elution time corresponds to PHBITC, for which a standard was available. It is also the dominant peak of the UV absorbance trace and is therefore easy to locate, as it has been enhanced to almost ten weight percent in the residual sample. Interestingly, PHBITC appears reactive in the electrospray source. Under positive ionization, a family of mass peaks are present that track with the UV peak, in comparing the absorbance trace to reconstructed ion chromatograms. However, elemental composition determinations are not straight-forward and suggest complex chemistry in the interface. For example, a highly mass deficient peak observed at m/z 392.0519 suggests clustering interactions. In contrast, m/z 107 is present as expected and is enhanced in hi-cone experiments (the p-OH benzyl cation, $C_7H_7O^+$. Finally, m/z 181.0959 fits N-Hydroxy-benzylamine, a decomposition product that makes chemical sense. In the negative ion mode, $[M^o + CF_3COO^-]$ was observed for PHBITC. Overall, the response factor of PHBITC is relatively low.

"9" - The formula of this compound indicates the putative plant metabolite "7", a methoxy derivative of IX, has undergone reaction via p-OH-benzyl substitution. By reconstructing ion traces for the $[M+Na]^+$ and $[M+K]^+$ forms, six minor isomers are seen in the HPLC trace.

Summary Overview and Conclusions - A graphical representation of the results is shown in Figure 9. Essentially, most of the ion current in the LC/MS traces is accounted for by isomers of a proposed natural metabolite 7, its further isomeric products formed by substitution of an equivalent of p-OH-benzyl (i.e., the family of compounds for 9), and the known decomposition product IV (HPLC peak 6) together with its two reaction products, 3 (p-OH benzyl substitution) and 2 (hydrolysis). By making assumptions regarding the UV absorbance of PHBITC, assayed at about ten weight percent, the estimated concentration of the major component 7 is around 0.1 weight percent.

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TABLE 1. Compounds reported in relation to the Brassicacae plant family.

COMPOUND	FORMULA	DBE - N/O/S CLASS
I. 4-hydroxybenzylglucosinolate anion	$C_{14}H_{18}NO_{10}S_2$	6.5-1/10/2
II. sinapine (quaternary cation)	$C_{16}H_{24}NO_5^+$	5.5-1/5/0
III. p-OH-benzyl mercaptan disulfide	$C_{14}H_{14}O_2S_2$	8- 0/2/2
IV. N,S-bis(p-hydroxybenzyl) dithiocarbamate	$C_{15}H_{15}NO_2S_2$	9-1/2/2
V. p-hydroxybenzyl amine	C_7H_9NO	4-1/1/0
VI. p-hydroxybenzylisothiocyanate	C_8H_7NOS	6-1/1/1
VII. N-hydroxybenzyl imine.	C_7H_7NO	5-1/1/0
VIII. p-hydroxybenzyldithiocarbamate	$C_8H_9NOS_2$	5-1/1/2
IX. Cyclobrassinin (CB)	$C_{12}H_{12}N_2OS_2$	8-2/1/2
X. Desmethoxy CB	$C_{11}H_{10}N_2S_2$	8-2/0/2
XI. Brassinin	$C_{12}H_{14}N_2OS_2$	7-2/1/2
XII. Desmethoxy Brassinin	$C_{11}H_{12}N_2S_2$	7/2/0/2
XIII. Sinalexin (S)	$C_{10}H_8N_2OS$	8-2/1/1
XIV. Desmethoxy Sinalexin	$C_9H_6N_2S$	8-2/0/2
XV. 4-hydroxybenzylthiohydroxamate-O-sulphonate	$C_8H_9NO_5S_2$	5-1/5/2
XVI. cyanadin sambubioside (pyrilium ion)	$C_{21}H_{21}O_8^+$	11.5-0/8/0
XVII. cyanadin (pyrilium ion)	$C_{15}H_{11}O_4^+$	10.5-0/4/0

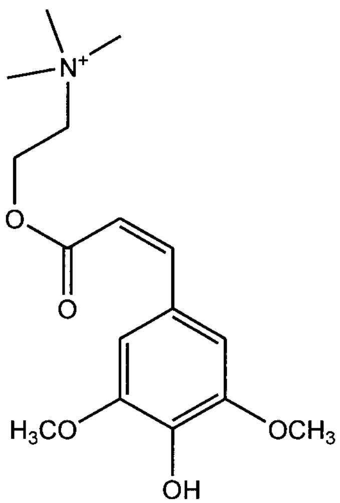
TABLE 2. Summary of Formulae Associated With HPLC Peaks in Figure 7.

HPLC Retention Time (min) and peak designation	M^0 (unless noted *) Formula and Accurate Mass	DBE and N/O/S Class	Comments- (All formulae #1 Isotope fit and within 2 PPM Unless Otherwise Noted)
1.60 / "Q"	*$C_{16}H_{24}NO_5^+$ 310.1649	5.5-1/5/0	Sinapine quaternary cation. Hi Cone - $N(CH_3)_3$ and CH_2CH_2O loss observed

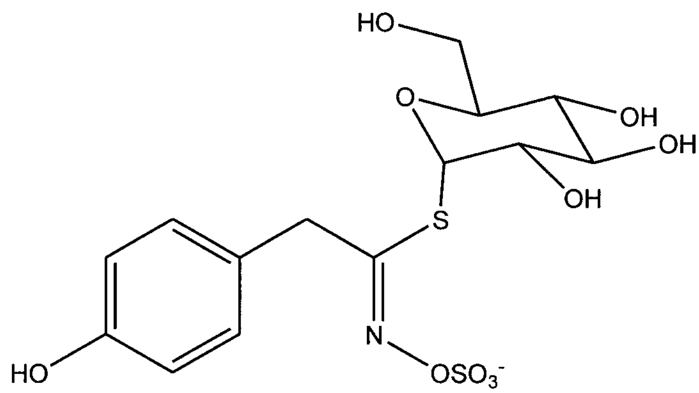
TABLE 2 Continued. Summary of Formulae Associated With HPLC Peaks in Figure 6.

3.01 / "1"	$C_{21}H_{21}NO_3$ 335.1521	12-1/3/0	Stoichiometrically 3x [p-OH Benzyl] on Nitrogen
4.87 / "2"	$C_{15}H_{15}NO_3S$ 289.0773	9-1/3/1	Derived From IV - replace C=S with C=O By Hydrolysis
4.92 / "A"	$*C_{28}H_{28}NO_4^+$ 442.2013	16-1/4/0	Stoichiometrically 4x [p-OH Benzyl] on Nitrogen (quaternary)
5.51 / "B"	$C_{19}H_{20}N_2O_2S$ 340.1245	11-2/2/1	minor peak; likely derived from brassin family and PHBITC reaction
5.71 / "C"	$C_{13}H_{14}N_2O_2S_2$ 294.0497	8-2/2/2	Isomer of "7" see below
5.81 / "3"	$C_{22}H_{22}N_2O_3S$ 394.1351	13-2/3/1	Derived From IV - Replace C=S with C=N-CH_2□□□□ (from reaction with V)
6.13 / "D"	$C_{13}H_{14}N_2O_2S_2$ 294.0497	8-2/2/2	Isomer of "7" see below
6.27 / "4"	$C_{14}H_{14}O_2S$ 246.0714	8-0/2/1	Stoichiometrically 2-[p-hydroxybenzyl] on sulfur; only observed in negative ion mode; intense formate adduct observed
6.78 / "5"	C_8H_7NOS 165.0248	6-1/1/1	p-OH benzylisothiocyanate - matches standard; ionizes in neg. ion as adduct with $CF3COO^-$; pos. ion spectrum implies reactive chemistry in ESI
7.11 / "6"	$C_{15}H_{15}NO_2S_2$ 305.0544	9-1/2/2	Formula matches IV - no standard. N,S-bis(p-hydroxybenzyl) dithiocarbamate
7.15 / "E"	$*C_{19}H_{39}N_2O_3^+$ 343.2955	2-2/3/0	Surfactant background -likely cocamidopropyl betaine quat.
7.76 / i-9	$C_{20}H_{20}N_2O_3S_2$ 400.0915	12-2/3/2	Isomer of "9" - see below
7.94 / "7"	$C_{13}H_{14}N_2O_2S_2$ 294.0497	8-2/2/2	Stoichiometrically replace H of Cyclobrassinin with methoxy
8.28 / i-7	$C_{13}H_{14}N_2O_2S_2$ 294.0497	8-2/2/2	"7" Isomer - see above
8.42 / "8"	$C_{12}H_{26}O_4S$ 266.1552	0-0/4/1	Lauryl sulfate surfactant background - only obs'd. in neg. ion; matches std.
8.74 / "i-9"	$C_{20}H_{20}N_2O_3S_2$ 400.0915	12-2/3/2	"9" Isomer - see below
9.30 / "F"	$C_{16}H_{33}NO_3$ 287.2460	1-1/3/0	Cocamide diethanolamine surfactant background peak
9.55 / "9"	$C_{20}H_{20}N_2O_3S_2$ 400.0915	12-2/3/2	Stoichiometrically replace H of "7" with p-hydroxybenzyl
10.71 / "G"	$C_{24}H_{21}N_3O_3S_3$ 495.0745	16-3/3/3	Stoichiometrically [3 x PHBITC]
10.78 / i-9	$C_{20}H_{20}N_2O_3S_2$ 400.0915	12-2/3/2	Stoichiometrically replace H of "7" with p-hydroxybenzyl

FIGURES

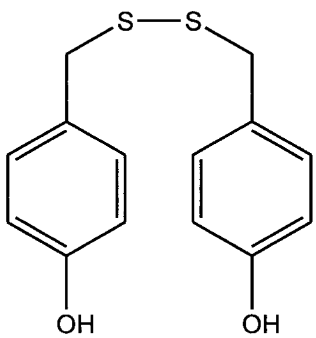


I.



II.

Figure 4. Structure of Sinalbin, a salt composed of the sinapine quaternary cation (I) and the 4-hydroxybenzyl glucosinolate anion.



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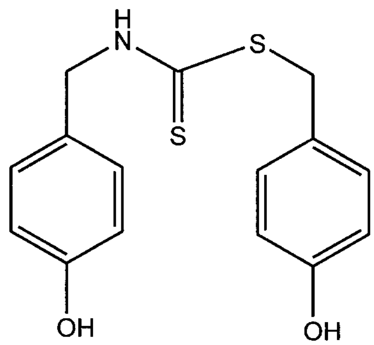

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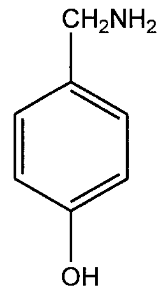

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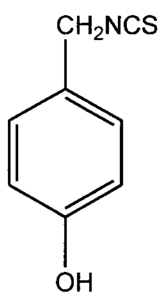

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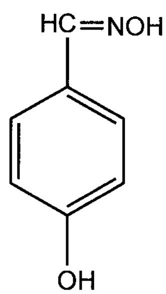
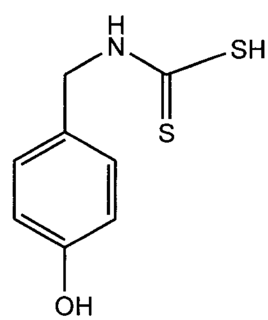
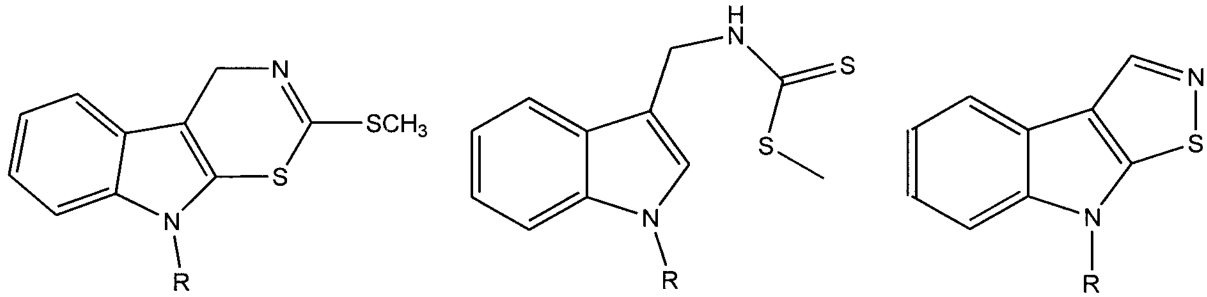


VII.



VIII.

Figure 5. Some reported decomposition products of Sinalbin.



IX. R = OCH_3
X. R = H

XI. R = OCH_3
XII. R = H

XIII. R = OCH_3
XIV. R = H

Figure 6. Structures of N-methoxy-cyclobrassinin (IX), cyclobrassinin (X), the acyclic derivatives N-methoxy-brassinin (XI) and brassinin (XII), and the isothiazole-ring-containing N-methoxy-sinalexin (XIII) and sinalexin (XIV).

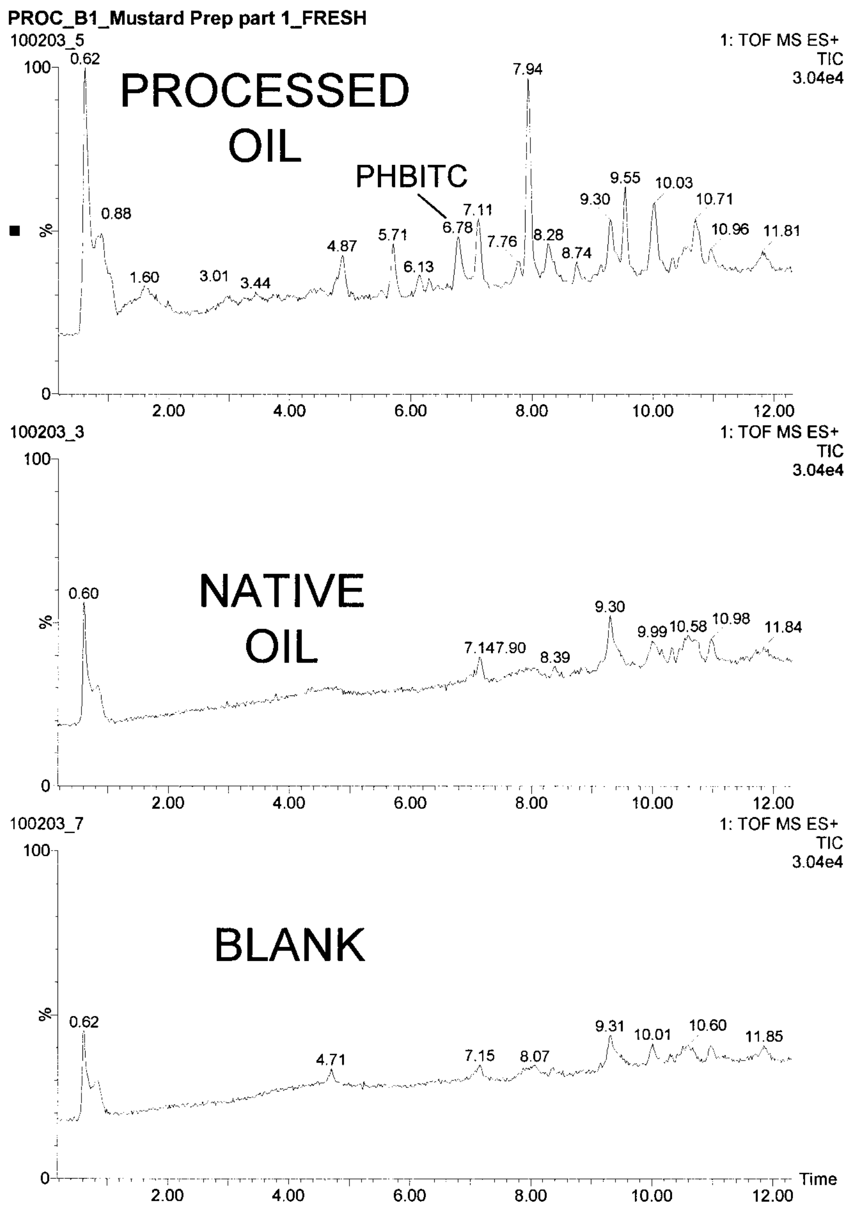


Figure 7. uHPLC-(+) ion ESI Tof-MS analysis of processed oil (top), cold pressed oil (middle), and a blank (bottom). The cold pressed oil did not show any differences vs. the blank.

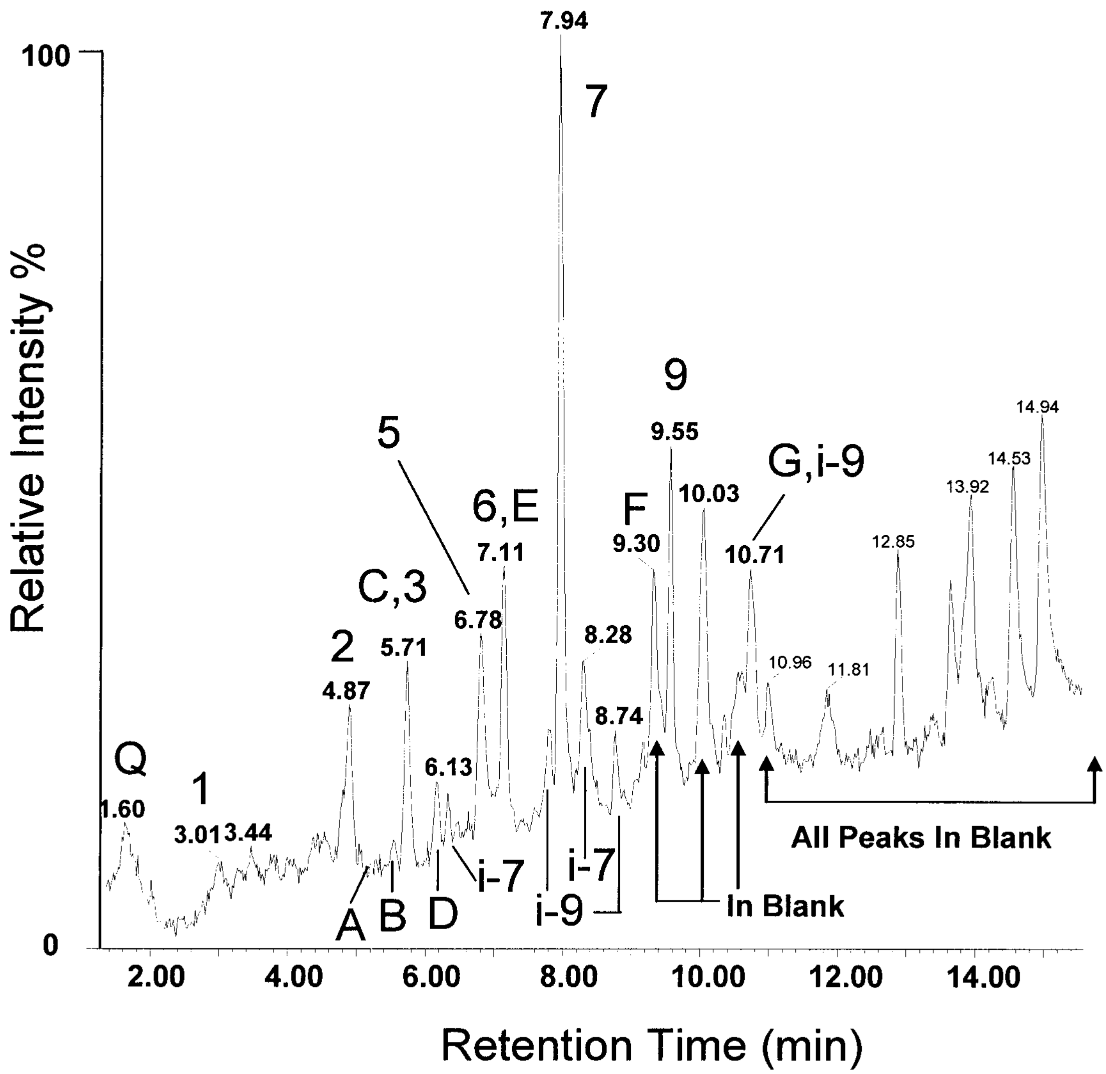


Figure 8. uHPLC-(+) ion ESI Tof-MS analysis of processed oil. The HPLC peak designations are by letters and Arabic numerals and correlate with Table 2.

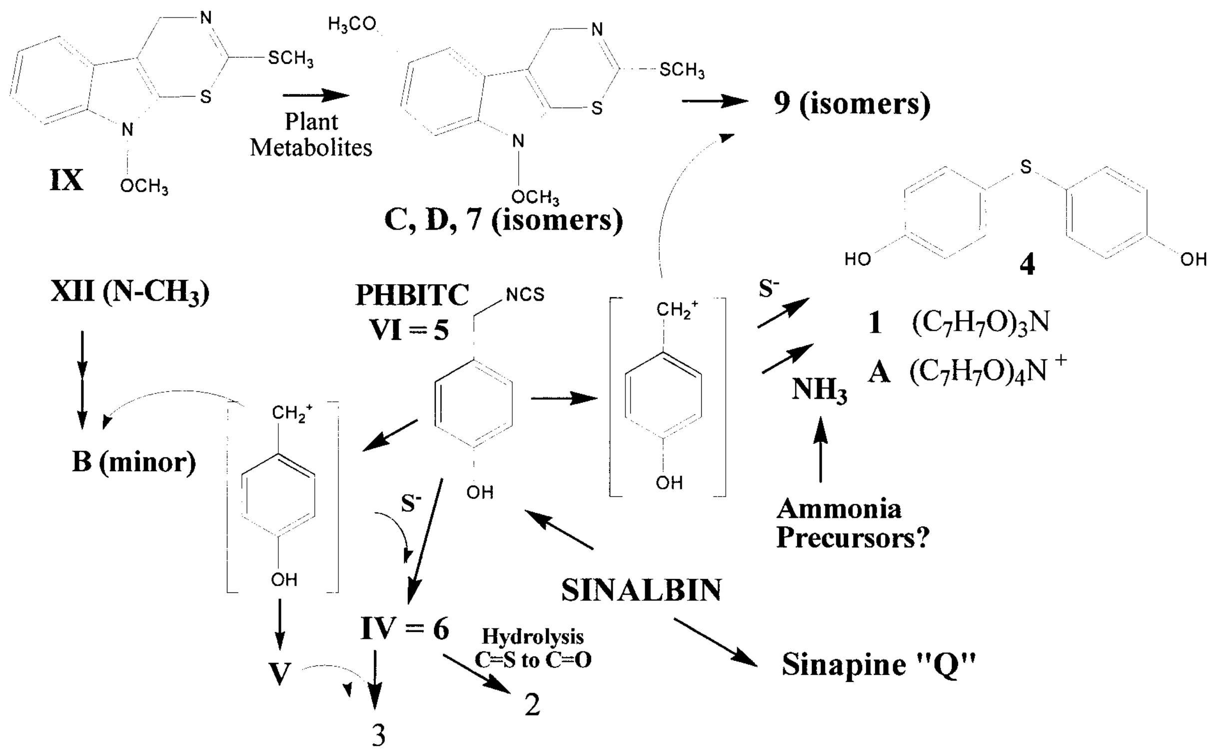


Figure 9. Hypothesized origins of the various compounds identified (Table 2). PHBITC = p-hydroxybenzylisothiocyante. Plant metabolites C,D, and 7 react with PHBITC which serves as a source of p-OH-benzyl (PHB) electrophile. Compounds 2, 6 & 4 parallel reported decomposition chemistry of PHBITC while 3 appears to be a further substitution reaction of the PHB electrophile with 2.

Hydrolytic reactions of 4HBITC in WMEO at various pHs' and a vegetable matrix

This report compiles the data from four hydrolysis experiments including three experiments done in buffer systems at pH 3.5, 6.0 and 9.0 and one experiment in Roasted Red Bell Pepper puree. The hydrolysis of the active component 4-hydroxybenzyl isothiocyanate (4HBITC) follows first order kinetics at all pH's with high correlation coefficients for the regression lines. However the profound influence of the hydroxyl ions on the hydrolytic reaction becomes apparent as low as pH 6.0 when the curve fits a second order reaction as well. The effect of OH^- is much more pronounced at pH 9.0. Thus the regression equations allow for the prediction of the final concentration of 4HBITC for known starting concentrations (Appendix - 1).

Under acidic pH conditions, possibly at pH < 5 hydrolysis reaction rates are the slowest with the rate of reaction about $1/10^{th}$ of that at pH 6.0. Above pH 6.0 reaction rates are rapid. Additionally at pH values up to about 6.0 the hydrolysis of 4HBITC causes a parallel increase in the concentration of 4-hydroxy-benzyl alcohol (4OHBA) without an increase in the p-cresol concentration. At alkaline pH values there is an increase in the concentrations of both the 4OHBA and p-cresol with the corresponding decrease in the concentration of 4HBITC. Also the absolute values of the concentration of 4OHBA is higher at both pH 3.5 and 6.0 compared to pH 9.0 where the final 4OHBA concentration is about ½ that of the more acidic pH's.

Roasted red bell pepper puree pH is typically 4.8-5.0 and the rate of hydrolysis of 4HBITC closely reflects the kinetics at that pH value. Solvent extraction of the white mustard essential oil containing roasted red bell pepper puree allows for the HPLC analysis of the remaining 4HBITC while the aqueous extracts even after dilution and filtration do not provide a clean enough baseline for meaningful analysis of 4OHBA or p-cresol.

Materials & Methods:

White mustard essential oil (WMEO) prepared at POS, Saskatoon, Saskatchewan, Canada was used as the starting material for the hydrolysis experiments. The concentration of 4-hydroxybenzyl isothiocyanate (4-HBITC) in the maltodextrin plated WMEO (WMEO-MD) was determined by HPLC. Based upon that determination, about 25.122 g of WMEO-MD was used to provide a 4-HBITC concentration of about 1000 µg/mL in the 200 mL buffer or vegetable puree sample used for the hydrolysis reactions described below.

Hydrolysis in buffers:
Citrate buffers of pH 3.5 and 6.0 (0.1M) were prepared by first making 0.2 M solutions of citric acid and trisodium citrate dehydrate and then titrating to the correct pH value and diluting 1:1 with distilled water and adjusting the final pH with the acid or salt solution. The buffers were stored refrigerated until use. Borate buffer pH 9.0 was prepared in the same manner using boric acid and sodium tetraborate decahydrate and stored refrigerated until use.
To start the hydrolysis experiments four separate 1 pint sized glass jars with lids were obtained and 200 mL of the buffers introduced into each of the jars. WMEO in maltodextrin (0.796% 4HBITC, about 25.122 g) was accurately weighed and introduced into the first jar. It was closed

and shaken well (10-15 sec.) by hand to disperse the maltodextrin. To obtain the time zero samples a 10 mL sample was transferred into a 25 mL separatory funnel followed by 5 mL ethyl acetate. The jar was closed again, re-shaken for 10-15 sec. and a 5 mL sample of the contents transferred to a pre-labeled glass jar. The glass jar was cooled in chilled water and immediately transferred into the -25°C freezer. The separatory funnel was shaken well and the ethyl acetate layers allowed to separate. The aqueous layer was allowed to drain via the stopcock and the ethyl acetate layer was poured into a glass bottle and closed with a stopper. The aqueous layer was re-extracted with a further 5 mL ethyl acetate. The ethyl acetate layers were pooled and dried over anhydrous sodium sulfate. After drying for about 4 hours the ethyl acetate solution containing the 4HBITC was transferred to a dry glass vial, labeled with the time and pH and stored at -25°C until analysis. This procedure was repeated for each of the other three jars. Thus at each time point each of the jars were sampled and an aqueous sample and an ethyl acetate extract of the solution were taken for a total of eight samples.
Sampling times for the pH 3.5 buffer hydrolysis reaction were: 0, 4, 8, 12, 24, 48, 72, 96, 120 and 312 (13 days) hours. Sampling times for the pH 6.0 buffer hydrolysis reaction were: 0, 4, 8, 12, 24, 48 and 72 hours. Sampling times for the pH 9.0 buffer hydrolysis reaction were: 0, 2, 4, 8, 12, 24, 48, and 72 hours.

Hydrolysis in vegetable puree:
Frozen roasted red bell pepper puree was thawed and 200.0 g (Lot received from ConAgra on 5/14/09: Notebook ref: HCF 243-057; pH = 4.965) was dispensed into 1 pint sized glass jars followed by approximately 25 .125 g of WMEO in maltodextrin (0.796% 4HBITC) weighed accurately. After premixing the maltodextrin into the roasted red bell pepper puree by hand, a ROTOSTAT probe (Rotostat Model XPLB-100, Admix, Manchester, NH) was introduced into the jar and stirred at medium speed to ensure homogeneity. A 25 mL plastic pipette (tip cut off for a larger orifice) was used to remove an approximately 17 mL sample. Of this a 10.0 g sample was transferred to a centrifuge tube and also an approximately 5 mL sample to a glass vial. The glass vial was labeled with the time and replicate number and stored in the -25°C freezer until analysis. Ethyl acetate (5 mL) was pipetted into the centrifuge tube and the contents mixed well using a vibrating shaker. Each of the four tubes resulting from the four jars was then centrifuged at 2700 rpm for 10 min. The top most layer containing ethyl acetate was transferred into a glass vial using a pasteur pipette and closed with a stopper. The ethyl acetate extraction was repeated once more and the pooled ethyl acetate layers were dried over anhydrous sodium sulfate for 3-4 hours. The dried ethyl acetate was then transferred to a dry glass vial, labeled with the time and replicate number and stored in the -25°C freezer until analysis.

HPLC analysis:
HPLC analysis entailed the use of two different HPLC systems. System one, used for normal phase chromatography, consisted of a Waters Alliance 2695, a Waters 996 PDA, and a Sedex 75 ELSD. System two, used for reverse phase chromatography, consisted of a Waters 2965 and a Waters 2996 PDA. A Betasil Diol-100 (4.6 mm id x 250 mm, 5 um) (Thermo Scientific, Cat no. 72605-254630) was used with the normal phase system. For the normal phase system, mobile phases A and B were hexane and isopropanol, respectively. A gradient method was used. The mobile phase composition was initially held at 5 % B for 2 minutes, it was then linearly increased to 25 % B over 15 minutes, then held at 25 % B for 0.5 minutes before returning to the

initial conditions. The following conditions were used with the normal phase system: flow rate = 1.3 mL/min, injection volume = 25 µL, detection wavelength -276 nm, ELSD gain = 10, ELSD nitrogen = 2.5, ELSD temperature = 45 °C, autosampler temperature = 15 °C.

A Waters Xbridge C18 column (4.6 mm id x 150 mm, 3.5 µM) (Waters, Cat no. 186003034) was used on the reverse phase system. Mobile phase A consisted of 0.1 % formic acid in water and mobile phase B consisted of 0.1 % formic acid in acetonitrile. A gradient method was used. The mobile phase composition was initially held at 2 % B for 2 minutes, then linearly increased to 95 % B over 12 minutes, then held at 95 % B for 0.5 minutes, before returning to the initial conditions. The following conditions were used: flow rate = 1.0 mL/min, injection volume = 25 µL, detection wavelength = 273 nm, autosampler temperature = 15°C.

Standards for the normal phase analysis were prepared by serial dilution in 20/80, ethyl acetate/hexane covering the range from 1 to 500 µg/mL. Normal phase standards contained 4HBITC, 4-OH benzyl alcohol, 4-OH benzyl cyanide, and p-cresol. The ethyl acetate samples were analyzed on the normal phase system. Samples were diluted 10:1 with 20/80 ethyl acetate/hexane. An aliquot of 3-OH Benzyl alcohol was added to each sample and standard as an internal standard (1.0 mg/mL solution in 1/1 ethyl acetate/hexane, add 20 µL/mL of sample or standard).

Standards for the reverse phase analysis were prepared by serial dilution in water over the range from 0.1 to 100 µg/mL. Reverse phase standards contained 4-OH benzyl alcohol, 4-OH benzyl cyanide, and p-cresol. The buffer samples were analyzed on the reverse phase system. The buffer samples were diluted 10:1 with water. The maltodextrin in the buffer samples is insoluble and the samples were filtered using filter vials with 0.45 µm PTFE membranes (Chrom Tech, Inc, Cat no. FV-2045). No filter bias was observed with the PTFE membrane filters. Note: A small amount of filter bias was noted with nylon membrane filters. The aqueous layer roasted red bell pepper puree has the consistency of tomato paste. These samples were weighed (~0.2 g), then suspended in water (10 mL), and filtered.

Chemicals:
4HBITC was isolated from a laboratory prepared WMEO sample as outlined in the qualitative portion of this report. Authentic samples of 4-hydroxybenzyl cyanide (4OHBCN) (Catalog no. H21101-50, lot no. 11829EH), 4-hydroxybenzyl alcohol (4OHBA) (catalog no. H20806-10G, lot no. 07409PH) and 3-OH benzyl alcohol (H20601-5G, LN 09621BH) obtained from Aldrich Chemical Co. An authentic sample of p-cresol (42429, lot no. 1399179) was obtained from Fluka. All solvents were HPLC grade or better.

Results and Discussion:

The normal phase method was used to quantitate the 4HBITC levels and the reverse phase method was used to quantitate p-cresol, 4-OH benzyl cyanide, and 4-OH-benzyl alcohol. A sample chromatogram of a standard on the normal phase system is shown in Figure 10. Sample chromatograms of the ethyl acetate extracts from the pH 3.5, pH 6, pH 9, and RRBP studies are shown in Figures 11-14, respectively. The identity of the compounds appearing at 10.1 min. in

Figure 13 is addressed on page 28. A sample chromatogram of a standard on the reverse phase system is shown in Figure 15. Sample chromatograms of the aqueous buffers from the pH 3.5, pH 6, pH 9, and RRBP studies are shown in Figures 16-19, respectively. On the reverse phase system such a large number of peaks were observed, that no meaningful results could be reported for the reverse phase analysis of the RRBP samples.

Hydrolysis in pH 3.5 citrate buffer was carried out in two stages as the 4-HBITC concentration was about 89 µg/mL after 5 days at 21°C. Another set of pH 3.5 buffer hydrolysis samples were prepared and allowed to react for 13 days at 21°C and sampled to ensure that the final concentration was closer to 0 µg/mL (actual 17.2 µg/mL). The data analysis includes the combined data.

Table 3 shows the raw HPLC data resulting from the normal phase and reverse phase analysis of the pH 3.5 buffer hydrolysis samples. This includes the concentrations of all the tracked constituents given in µg/mL (ppm) and also the concentration of 4HBITC in m mol L^{-1} . Table 3 also shows the natural logarithm of the concentration of 4-HBITC and the inverse of the concentration of 4-HBITC used for curve fitting to determine order of reaction including the correlation coefficients. Figure 20 shows the average concentration vs. time plots for all of the tracked components 4-hydroxybenzyl isothiocyanate (4HBITC); 4-hydroxybenzyl alcohol (4OHBA); 4-hydroxybenzyl cyanide (4OHBCN) & p-cresol. The increase in the 4OHBA concentration seems to closely parallel the decrease in concentration of the 4HBITC at this pH. The 4OHBCN and p-cresol concentrations seem to be much less dependent on time showing an almost constant amount over the 13 day period. Figure 21 shows the plot of the natural logarithm of the concentration of 4HBITC vs. time and the regression line obtained by statistical analysis of the data. The regression line fits the data very well as seen by the high R^2 value of 0.98. This shows that the hydrolysis of 4HBITC is a first order reaction given by the equation LN [4HBITC] = -0.017588 x time + 1.502754. The intercept is the natural logarithm of the time zero concentration of 4HBITC and when the constant slope factor is used, allows the prediction of the concentration of 4HBITC at a given time for a given starting concentration.

The above data shows that the 4HBITC is relatively stable in acidic pHs' even at room temperature. This property may be used to provide a microbial reduction in naturally acidic or intentionally acidified products prior to exposing them to a thermal treatment, thus allowing a much milder thermal treatment which may well result in the retention of more of the desired sensory attributes of these products.

Hydrolysis in pH 6.0 buffer was much quicker as seen by the data in Table 4 and Figure 22. Here the 4HBITC is completely hydrolyzed between 24 and 48 hours. Interestingly the p-cresol concentration does not increase very much as a consequence of the disappearance of 4HBITC as seen in Figure 22. The regression line of the natural logarithm of the 4HBITC concentration fits the data very well showing similar first order reaction kinetics with much enhanced slope of -0.1883 s^{-1} (Figure 23). However as seen in Table 5, the regression coefficients for the natural log of the concentration of 4-HBITC and the inverse of the concentration of 4-HBITC are very close to each other, showing that the hydrolysis reaction seems to shift to a second order reaction with the increase in pH. Knowing that the hydrolysis of 4-HBITC is pH dependant we may infer that at some pH between 3.5 and 6.0 the concentration of available OH^- ions becomes a powerful influence on reaction order.

Hydrolysis in pH 9.0 buffer is almost instantaneous as seen by the very low concentration of 4HBITC at time zero sampling (Table 6 and Figure 24). The actual time between the addition of the WMEO-MD and the ethyl acetate addition to extract the 4HBITC would have been about 5 minutes at the outset. Within that short a period of time > 90% of the 4HBITC is hydrolyzed. For kinetics analysis a hypothetical concentration of 0.01 µg/mL was used at the 2 hour time point and the hypothetical regression curve showed a much higher slope -3.5445 s^{-1}, showing a very quick hydrolysis reaction at alkaline pHs'.

As proposed (Ekanayake, 2006) the alkaline hydrolysis reaction of 4HBITC is very fast and based upon the large increase in reaction rate from pH 6.0 to pH 9.0, we can infer that the concentrations of both 4HBITC and OH^- are the factors involved in the second order reaction at pH 9.0. Additionally the concentration of p-cresol as well as 4OHBA does increase with the disappearance of 4HBITC (Table 6 and Figure 24). However the absolute concentration of 4OHBA shows a reduction from about 563 µg/mL at pH 3.5 to about 378 µg/mL at pH 9.0 showing that the reaction mechanism does change at alkaline pHs'. This alkaline condition does not exist in most foods except perhaps in Egg beaters® where the pH is about 7.9 - 8.0 thus possibly reducing the rate of hydrolysis.

The compound giving a peak (Peak A) at 10.1 min. in the HPLC chromatogram in Figure 13 seems to be an unstable intermediate formed during the alkaline hydrolysis of 4HBITC. Tracking its presence over the 48 hour period of hydrolysis confirmed its instability by its disappearance and by the corresponding increase in the concentration of 4OHBA (Figure 25). Additionally based on peak area measurements the decrease in the concentration of the 'A' corresponds very well to an increase in the concentration of 4OHBA (Figure 26). Kawakishi and Muramatsu (1966) had observed such compounds which are unstable under alkaline conditions during their work on the hydrolysis of sinalbin in model systems as well as in moistened mustard powder.

Roasted red bell pepper puree has a pH of about 4.9-5.1 depending on the harvest season. This particular lot has a pH of 4.97. As such we could expect the 4HBITC to hydrolyze at a rate intermediate to pH 3.5 and pH 6.0. Table 7 and Figure 27 show the actual data and the plotted curve for the concentration of 4HBITC with time in roasted red bell pepper puree. On taking the 72 hour sample a noticeable escape of gas from one of the jars indicative of microbial spoilage activity prevented extending out the experiment to observe the further hydrolysis of 4HBITC. This is to be expected as the frozen roasted red bell pepper puree had not been blanched and the roasting step is at most an uneven thermal treatment thus preserving the propensity for spoilage. The ethyl acetate extracts made from the roasted red bell pepper puree were red colored and did not cause any unusual problems to do with HPLC analysis (Figure 14). The same did not hold true for the analysis of the aqueous extracts. Even after dilution (1:10) and filtration, the HPLC analysis proved to be difficult and the data generated were not truly indicative of the actual concentrations of the resulting compounds because of major baseline unresolved peaks and humps (Figure 19).

A comparison of the regression lines describing the kinetics of the hydrolysis reaction is given in Figure 28. This shows the dramatic increase in the rate of the hydrolysis reaction of 4HBITC with increasing pH of the medium. This is also perhaps indicative of the increasing importance of the hydroxyl ions in the hydrolysis reaction changing it from a typical first order reaction at low pH, involving only the concentration of 4HBITC, to possibly a second order reaction at higher pH with the involvement of OH^- as well.

Tables & Figures.

Table 3. Raw HPLC data, hydrolysis of 4HBITC at pH 3.5, 21°C.

Time (hrs.)	[4HBITC] (μg/mL)	[4OHBA] (μg/mL)	[4OHBCN] (μg/mL)	[p-cresol] (μg/mL)	[4HBITC] (m mol/L)	Ln [4HBITC]	$[4HBITC]^{-1}$
0	849.5	54.3	4.65	0.00	5.1424	1.6375	0.1945
0	853.0	51.9	4.30	0.00	5.1632	1.6416	0.1937
0	789.7	57.1	4.41	0.00	4.7804	1.5645	0.2092
0	686.6	65.3	4.44	0.00	4.1561	1.4246	0.2406
4	441.9	112.1	5.10	0.00	2.6747	0.9839	0.3739
4	733.9	116.7	4.49	0.00	4.4423	1.4912	0.2251
4	727.2	120.2	5.06	0.00	4.4021	1.4821	0.2272
4	747.9	130.6	4.84	0.00	4.5269	1.5100	0.2209
8	713.2	165.2	4.56	0.00	4.3172	1.4626	0.2316
8	660.8	172.0	3.48	0.00	3.9996	1.3862	0.2500
8	652.8	172.7	4.65	0.00	3.9515	1.3741	0.2531
8	673.7	184.4	4.86	0.00	4.0777	1.4055	0.2452
12	620.1	219.8	4.65	0.00	3.7535	1.3227	0.2664
12	608.7	219.6	4.59	0.00	3.6846	1.3042	0.2714
12	613.5	231.2	3.94	0.00	3.7135	1.3120	0.2693
12	604.4	240.7	4.66	0.00	3.6584	1.2970	0.2733
24	479.1	307.8	4.71	0.00	2.9000	1.0647	0.3448
24	480.9	315.6	4.43	0.00	2.9111	1.0685	0.3435
24	453.2	319.7	4.81	0.00	2.7433	1.0092	0.3645
24	485.5	325.9	4.52	0.00	2.9390	1.0781	0.3402
48	285.3	412.9	4.78	0.00	1.7271	0.5464	0.5790
48	326.8	416.7	4.63	0.00	1.9781	0.6821	0.5055
48	265.9	415.6	4.75	0.00	1.6094	0.4759	0.6213
48	299.6	415.2	4.63	0.00	1.8135	0.5952	0.5514
72	219.6	472.2	4.45	4.26	1.3292	0.2846	0.7523
72	221.8	474.2	5.12	3.67	1.3426	0.2946	0.7448
72	211.4	472.9	4.98	0.00	1.2796	0.2466	0.7815
72	186.7	473.5	4.74	3.26	1.1302	0.1224	0.8848
96	136.7	514.9	4.63	5.32	0.8273	-0.1896	1.2088
96	146.2	517.1	4.78	5.06	0.8852	-0.1219	1.1297
96	149.1	507.1	4.56	5.06	0.9025	-0.1026	1.1081
96	151.0	517.0	4.67	5.23	0.9140	-0.0900	1.0941
120	79.7	537.3	4.43	7.37	0.4822	-0.7294	2.0739
120	97.5	550.8	4.94	4.56	0.5904	-0.5269	1.6937
120	100.0	538.2	4.58	6.05	0.6056	-0.5016	1.6514
120	80.3	549.2	4.74	4.96	0.4862	-0.7212	2.0568

Table 4. Raw HPLC data, hydrolysis of 4HBITC at pH 6.0, 21°C.

Time (hrs.)	[4HBITC] µg/mL	[4OHBA] µg/mL	[4OHBC] µg/mL	p-cresol µg/mL	[4HBITC] (m mol L^{-1})	LN[4HBITC]	1/[4HBITC] (m mol^{-1} L)
0	831.0	367.2	21.47	4.38	5.0302	1.6155	0.1988
0	783.8	387.4	23.07	4.79	4.7447	1.5570	0.2108
0	816.5	408.1	22.98	4.55	4.9425	1.5979	0.2023
0	825.3	364.6	20.67	4.68	4.9955	1.6085	0.2002
4	188.2	464.1	21.70	6.57	1.1389	0.1301	0.8780
4	186.5	501.6	23.81	7.72	1.1289	0.1212	0.8858
4	178.7	470.6	21.63	6.23	1.0816	0.0784	0.9246
4	186.1	508.0	23.85	7.03	1.1266	0.1192	0.8876
8.5	65.6	543.8	22.17	7.66	0.3971	-0.9235	2.5180
8.5	63.2	510.8	20.52	6.66	0.3827	-0.9606	2.6132
8.5	57.9	552.8	23.53	7.87	0.3502	-1.0493	2.8557
8.5	55.1	560.8	21.92	7.74	0.3336	-1.0978	2.9977
12	24.0	578.6	20.93	8.12	0.1452	-1.9297	6.8875
12	23.8	540.0	20.36	7.45	0.1439	-1.9383	6.9472
12	22.2	554.5	21.19	7.59	0.1346	-2.0056	7.4303
12	24.0	578.4	21.93	8.37	0.1454	-1.9285	6.8795
24	9.6	552.7	17.95	6.91	0.0578	-2.8503	17.2934
24	6.9	598.7	17.64	8.26	0.0417	-3.1770	23.9739
24	6.7	606.8	18.58	8.12	0.0404	-3.2079	24.7275
24	7.2	612.2	19.11	8.06	0.0436	-3.1319	22.9164
48	0.0	549.7	18.42	7.48			
48	0.0	595.4	20.83	8.27			
48	0.0	596.1	20.98	7.42			
48	0.0	595.3	20.77	7.50			
72	0.0	566.5	17.38	6.74			
72	0.0	560.3	16.28	7.16			
72	0.0	612.9	18.07	7.24			
72	0.0	610.5	17.50	8.15			
96	0.0	615.7	17.38	7.33			
96	0.0	611.2	16.57	7.93			
96	0.0	616.2	16.77	8.47			
96	0.0	612.8	16.18	8.30			

Table 5. Correlation coefficients of regression lines of derived variables vs. time used for reaction order determination

	Correlation coefficient	
Derived variable	pH 3.5	pH 6.0
LN [4HBITC]	0.98	0.97
1/[4HBITC]	0.91	0.96

Table 6. Raw HPLC data, hydrolysis of 4HBITC at pH 9.0, 21°C.

Time (hrs.)	[4HBITC] (µg/mL)	[4OHBA] (µg/mL)	[4OHBCN] (µg/mL)	p-cresol (µg/mL)	[4HBITC] (m mol L^{-1})	LN[4HBITC]	1/[4HBITC] (m mol^{-1} L)
0	14.6	240.0	3.28	4.37	0.0886	-2.4232	11.2821
0	7.64	261.3	2.83	4.96	0.0463	-3.0734	21.6151
0	16.0	284.2	3.93	5.32	0.0967	-2.3363	10.3434
0	11.6	262.1	3.25	5.43	0.0699	-2.6602	14.2997
2	0.0	293.7	3.87	8.15	0.0001	-9.7124	16520
2	0.0	280.1	3.74	7.30	0.0001	-9.7124	16520
2	0.0	281.0	3.96	7.31	0.0001	-9.7124	16520
2	0.0	294.4	4.45	8.02	0.0001	-9.7124	16520
4	0.0	314.6	3.99	10.81			
4	0.0	329.9	4.10	10.64			
4	0.0	316.9	4.12	10.58			
4	0.0	319.5	4.31	10.51			
8	0.0	347.5	4.51	14.13			
8	0.0	350.0	4.00	14.31			
8	0.0	346.3	4.07	14.03			
8	0.0	350.5	4.75	13.78			
12	0.0	359.1	4.64	16.01			
12	0.0	362.7	4.41	16.66			
12	0.0	359.8	4.77	16.29			
12	0.0	361.6	4.20	16.39			
24	0.0	367.7	4.75	18.62			
24	0.0	368.5	4.83	18.96			
24	0.0	360.8	4.62	18.85			
24	0.0	363.0	4.62	18.52			
48	0.0	376.1	5.04	21.98			
48	0.0	382.3	4.44	22.59			
48	0.0	377.3	4.61	22.51			
48	0.0	379.5	4.49	22.04			

t=2 hrs point input as 0.01 µg/mL for calculation purposes.

Table 7. Raw HPLC data for concentration of 4HBITC added to Roasted Red Bell Pepper puree, natural pH 4.97, 21°C.

Time (hrs.)	[4HBITC] (μg/mL)	[4HBITC] (m mol L^{-1})	LN[4HBITC]	1/[4HBITC]
0	479.5	2.9024	1.0655	0.3445
0	728.6	4.4103	1.4839	0.2267
0	635.7	3.8481	1.3476	0.2599
0	866.9	5.2475	1.6577	0.1906
4	855.8	5.1804	1.6449	0.1930
4	627.0	3.7952	1.3337	0.2635
4	686.6	4.1562	1.4246	0.2406
4	687.7	4.1629	1.4262	0.2402
8	680.4	4.1188	1.4156	0.2428
8	574.8	3.4795	1.2469	0.2874
8	553.6	3.3507	1.2092	0.2984
8	511.2	3.0943	1.1296	0.3232
12	576.6	3.4903	1.2500	0.2865
12	510.7	3.0913	1.1286	0.3235
12	457.3	2.7681	1.0182	0.3613
12	539.4	3.2651	1.1833	0.3063
24	320.4	1.9393	0.6623	0.5157
24	353.6	2.1403	0.7609	0.4672
24	287.8	1.7421	0.5551	0.5740
24	292.5	1.7704	0.5712	0.5648
48	175.2	1.0603	0.0586	0.9431
48	184.8	1.1186	0.1120	0.8940
48	183.9	1.1134	0.1074	0.8981
48	151.2	0.9154	-0.0884	1.0925
72	103.9	0.6292	-0.4633	1.5893
72	110.2	0.6673	-0.4045	1.4986
72	105.9	0.6411	-0.4446	1.5599
72	90.5	-0.6024	1.8265	0.5475

Figure 10. Chromatogram of a 50 µg/mL standard on the normal phase system.

Compound	Retention Time (min)
p-cresol	5.9
p-HBITC (4HBITC)	8.8
4-OH benzyl cyanide	12.7
3-OH benzyl alcohol	13.8
4-OH benzyl alcohol	15.3

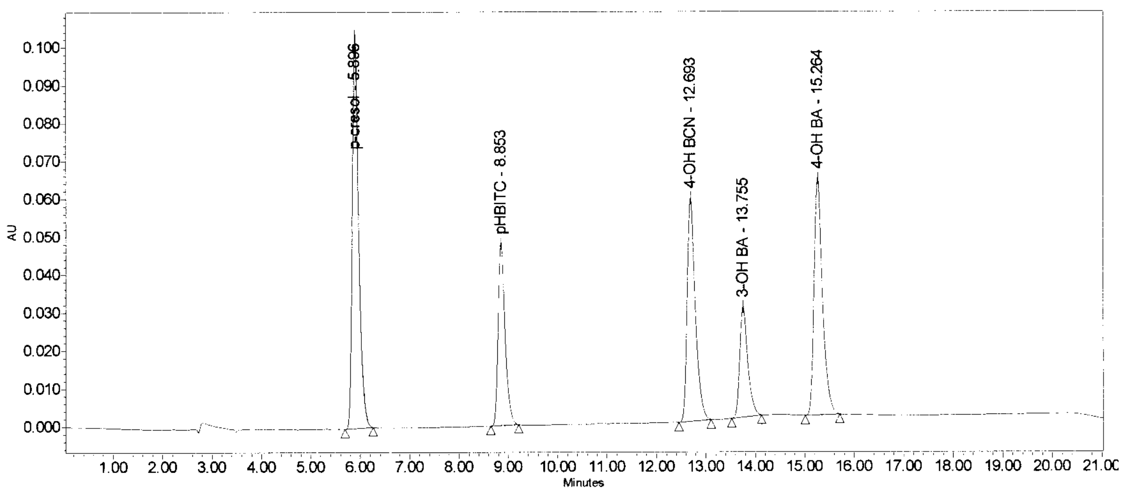


Figure 11. Normal phase chromatogram of the ethyl acetate extract for the 12 hour time point for the
pH 3.5 buffer.

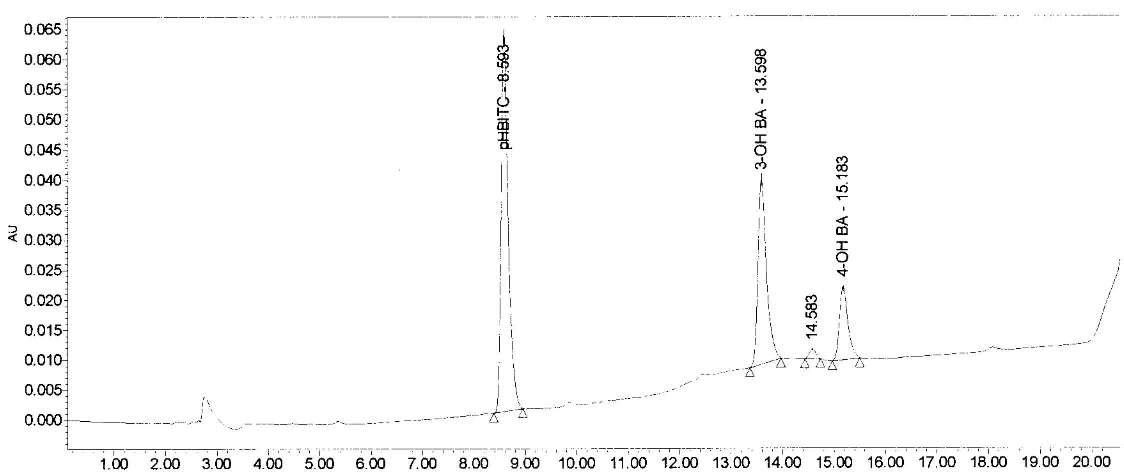


Figure 12. Normal phase chromatogram of the ethyl acetate extract for the 12 hour time point for the
pH 6 buffer.

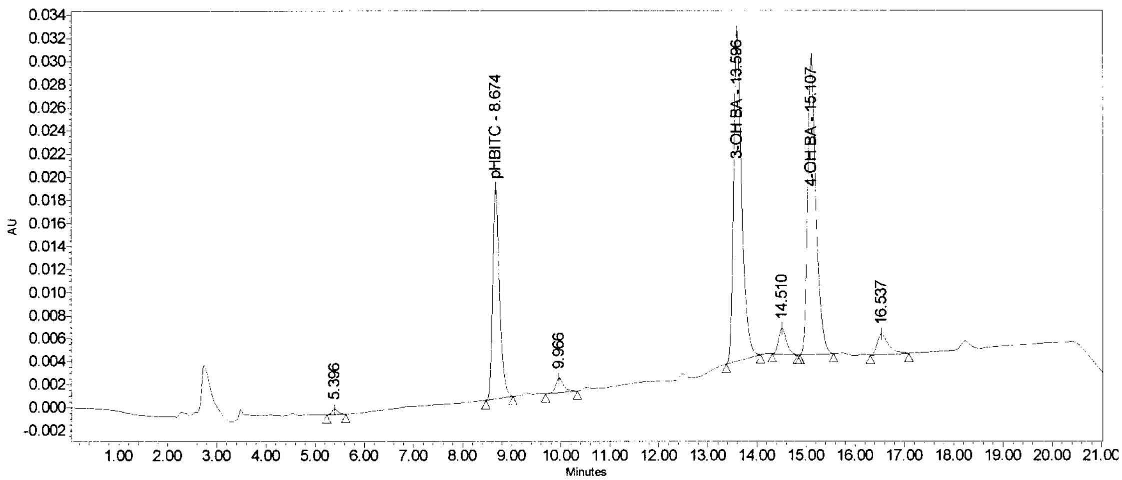


Figure 13. Normal phase chromatogram of the ethyl acetate extract for the 0 hour time point for the
pH 9 buffer.

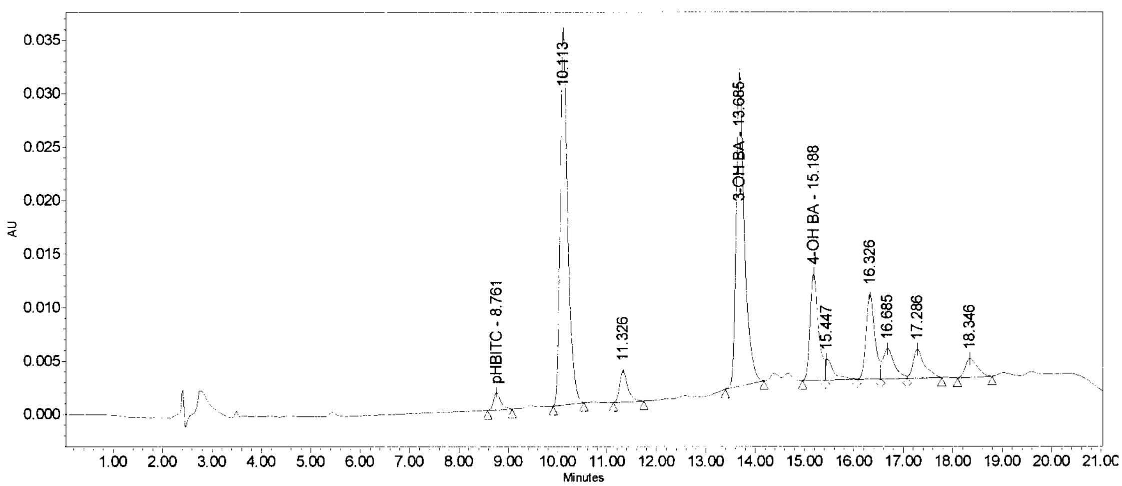


Figure 14. Normal phase chromatogram of the ethyl acetate extract for the 0 hour time point for the
Roasted red bell pepper puree

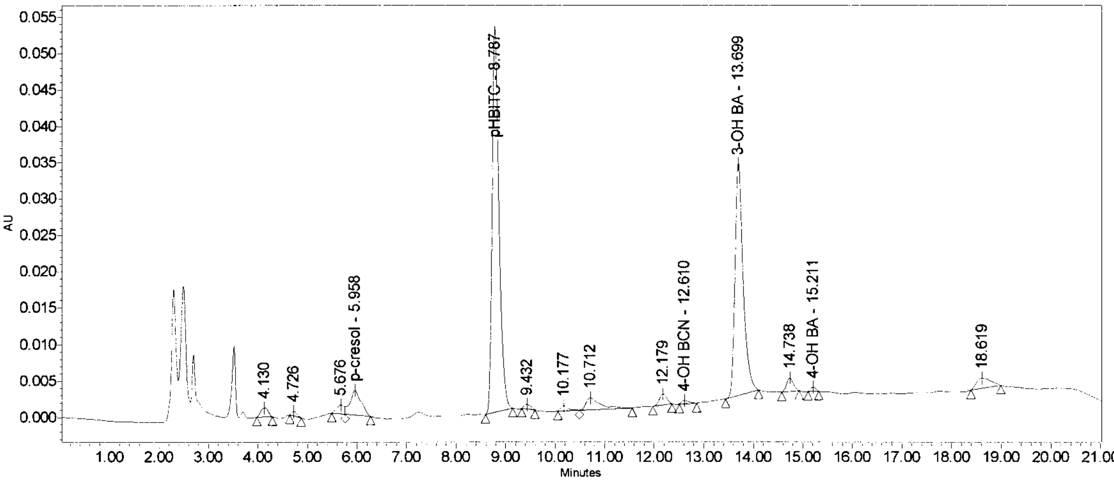


Figure 15. Chromatogram of a 50 μg/mL standard on the reverse phase system.

Compound	Retention Time (min)
4-OH benzyl alcohol	6.3
4-OH benzyl cyanide	8.5
p-cresol	10.1
p-HBITC	11.4

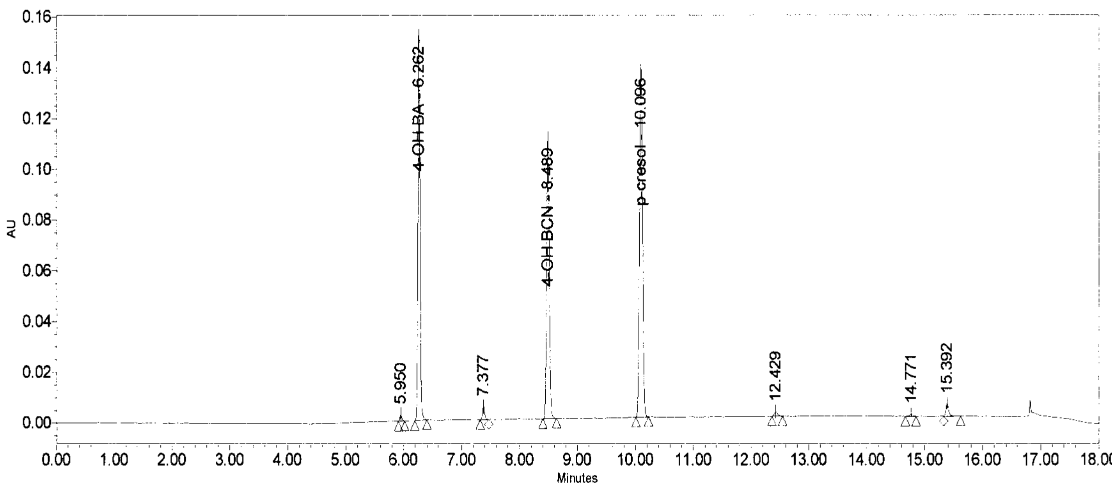


Figure 16. Reverse phase chromatogram of the pH 3.5 buffer at the 24 hour time point.

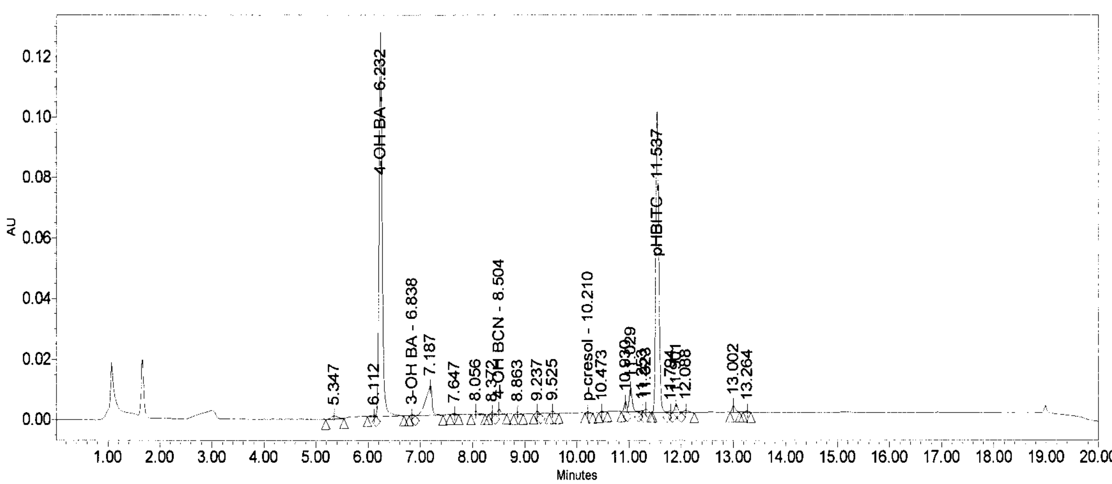


Figure 17. Reverse phase chromatogram of the pH 6 buffer at the 4 hour time point.

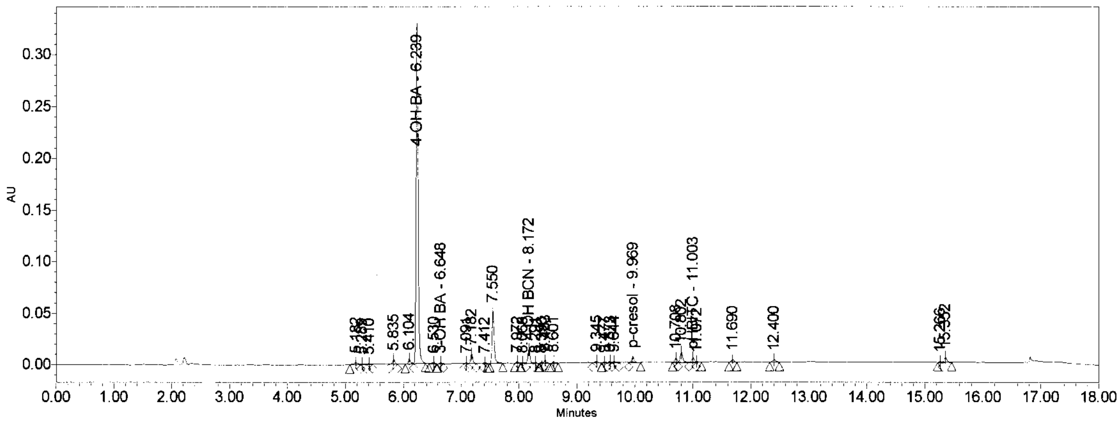


Figure 18. Reverse phase chromatogram of the pH 9 buffer at the 2 hour time point.

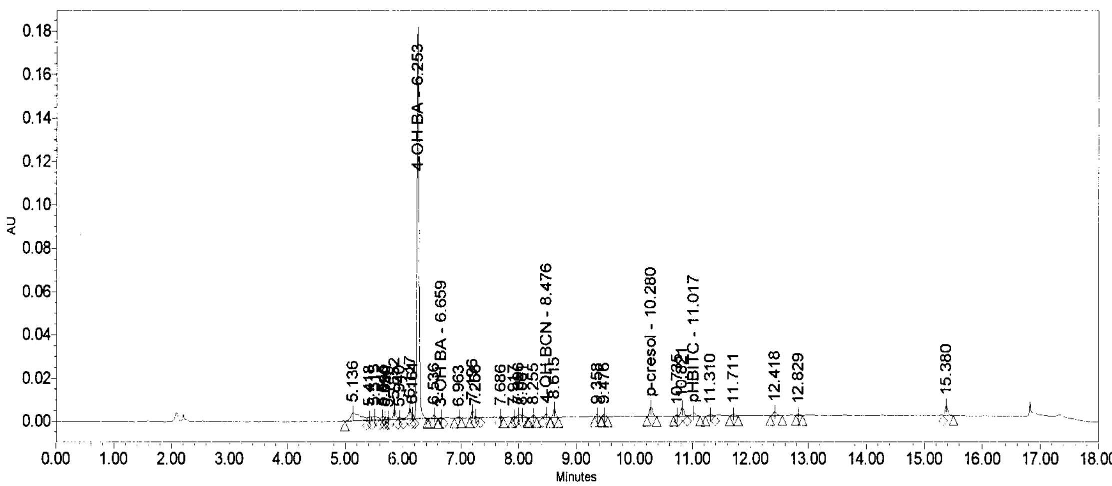


Figure 19. Reverse phase chromatogram of the aqueous layer of the roasted red bell pepper puree at the 12 hour time point.

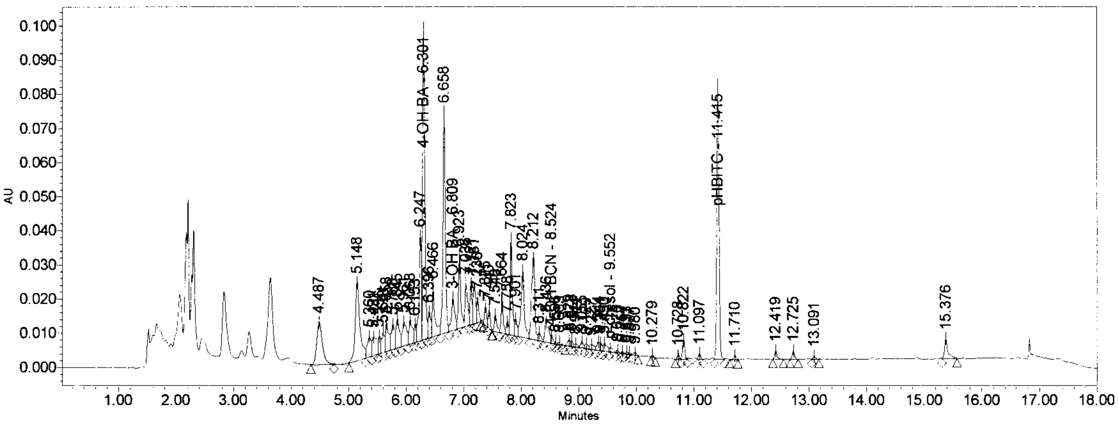
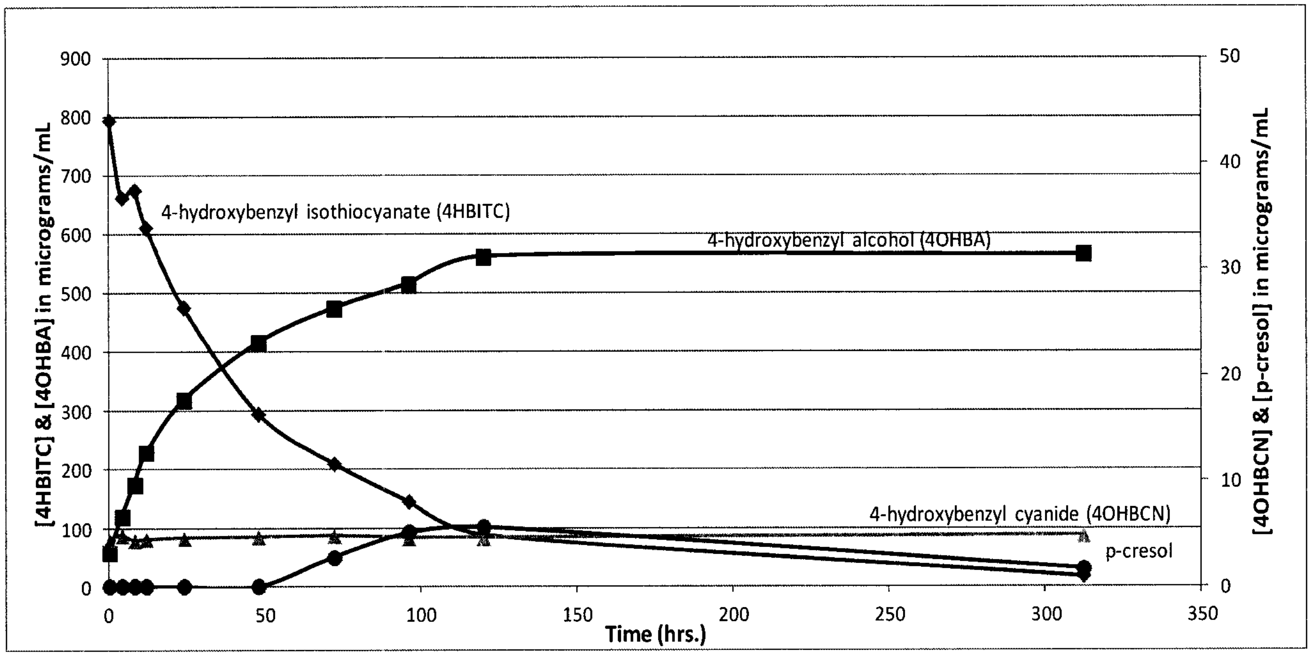


Figure 20. Concentration of 4HBITC, 4OHBA, 4OHBCN and p-cresol with time, pH 3.5, 21°C. Average of 4 determinations per time point.



Note: Data at time 312 hours (13 days) from a parallel experiment included in chart.

Figure 21. Natural logarithm of the concentration of 4HBITC with time; pH 3.5, 21°C

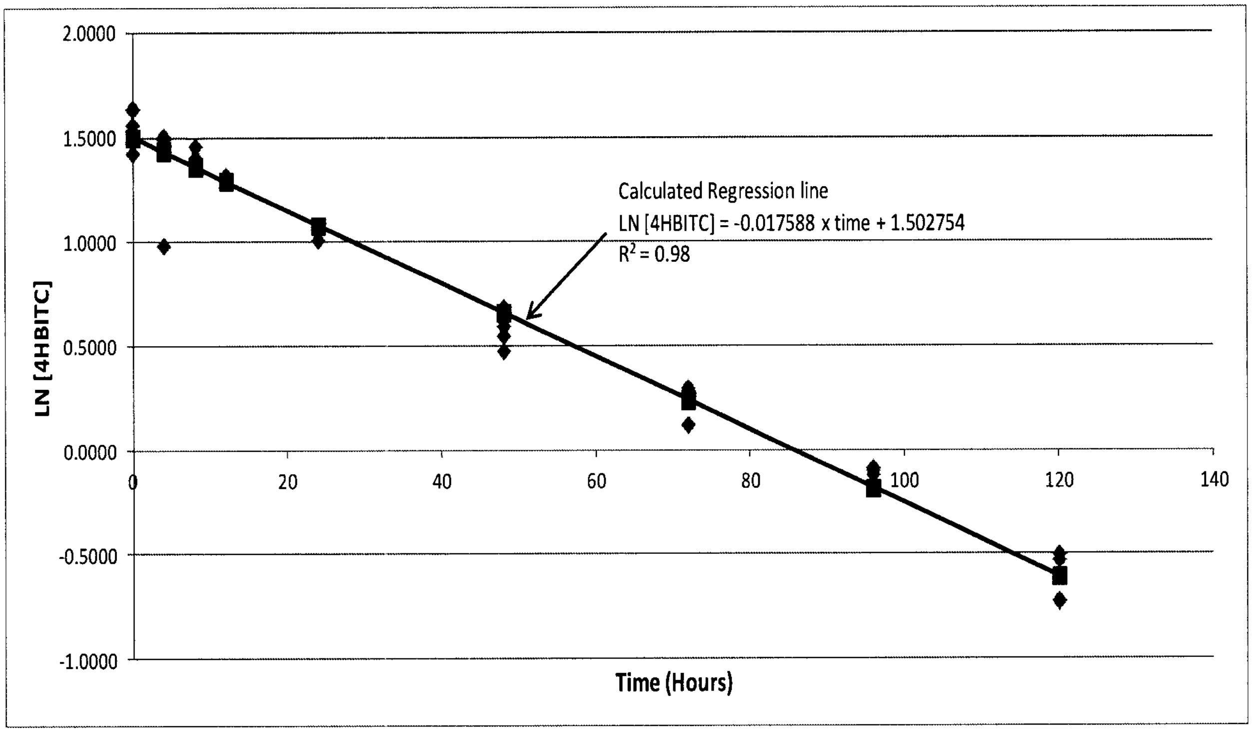


Figure 22. Concentration of 4HBITC, 4OHBA, 4OHBCN and p-cresol with time, pH 6.0, 21°C. Average of 4 determinations per time point.

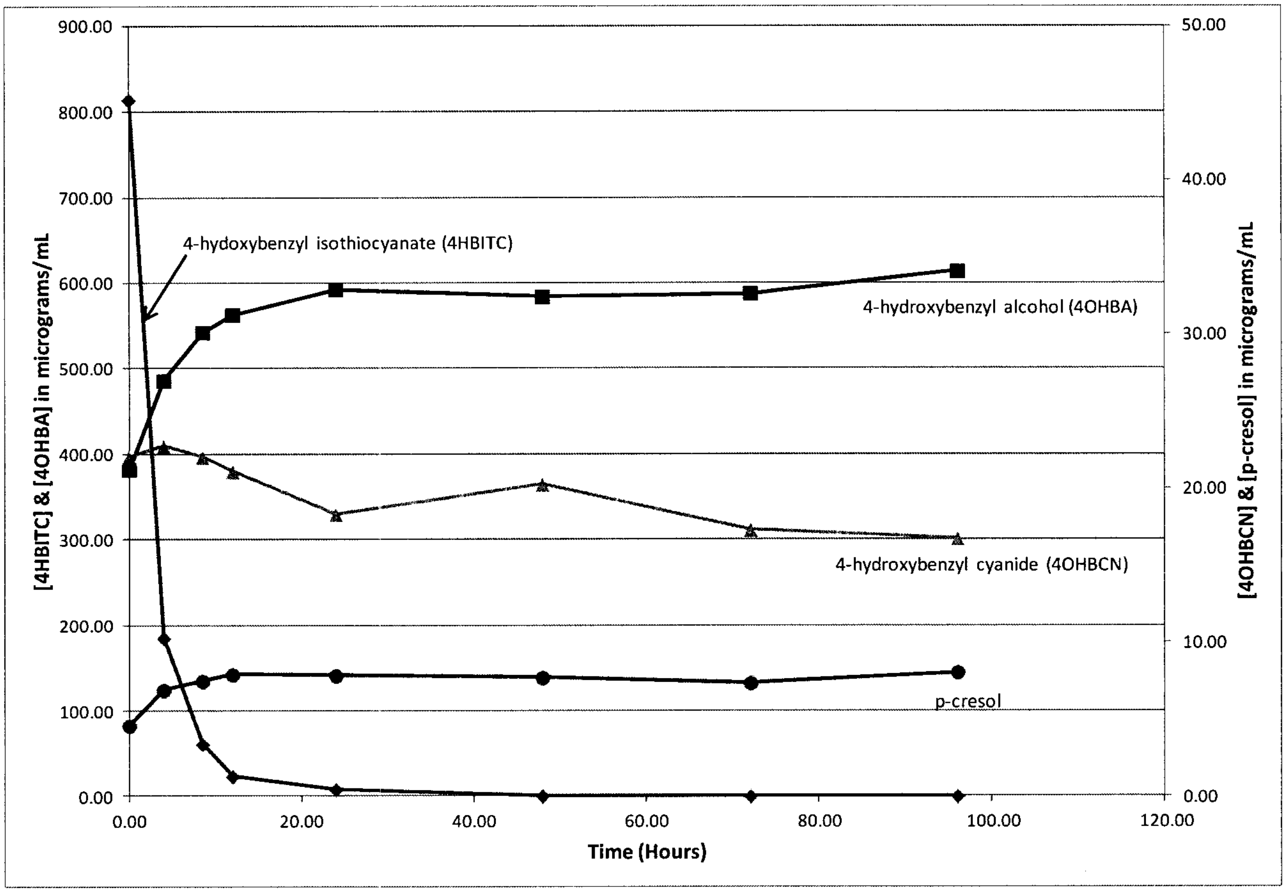


Figure 23. Natural logarithm of the concentration of 4HBITC with time; pH 6.0, 21°C

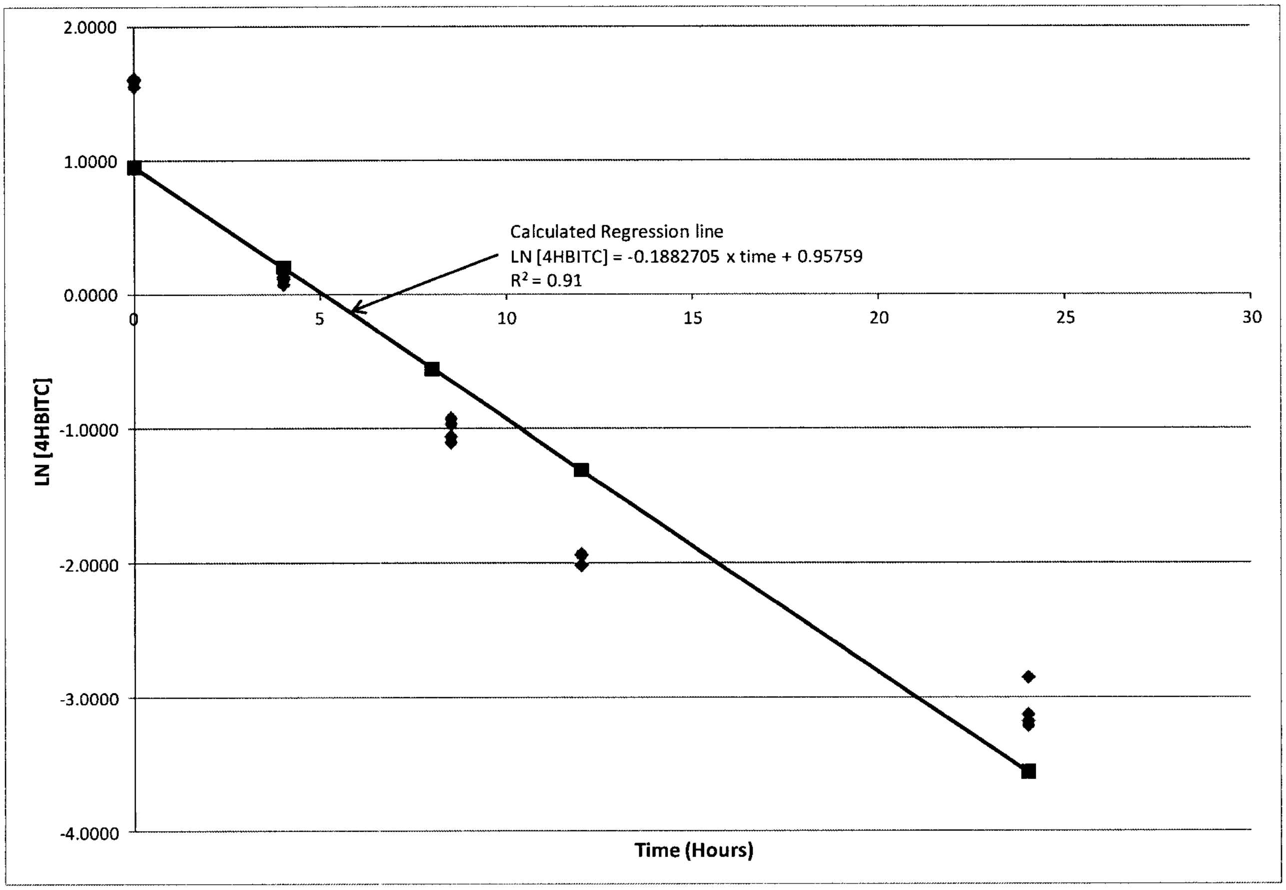


Figure 24. Concentration of 4HBITC, 4OHBA, 4OHBCN and p-cresol with time, pH 9.0, 21°C. Average of 4 determinations per time point.

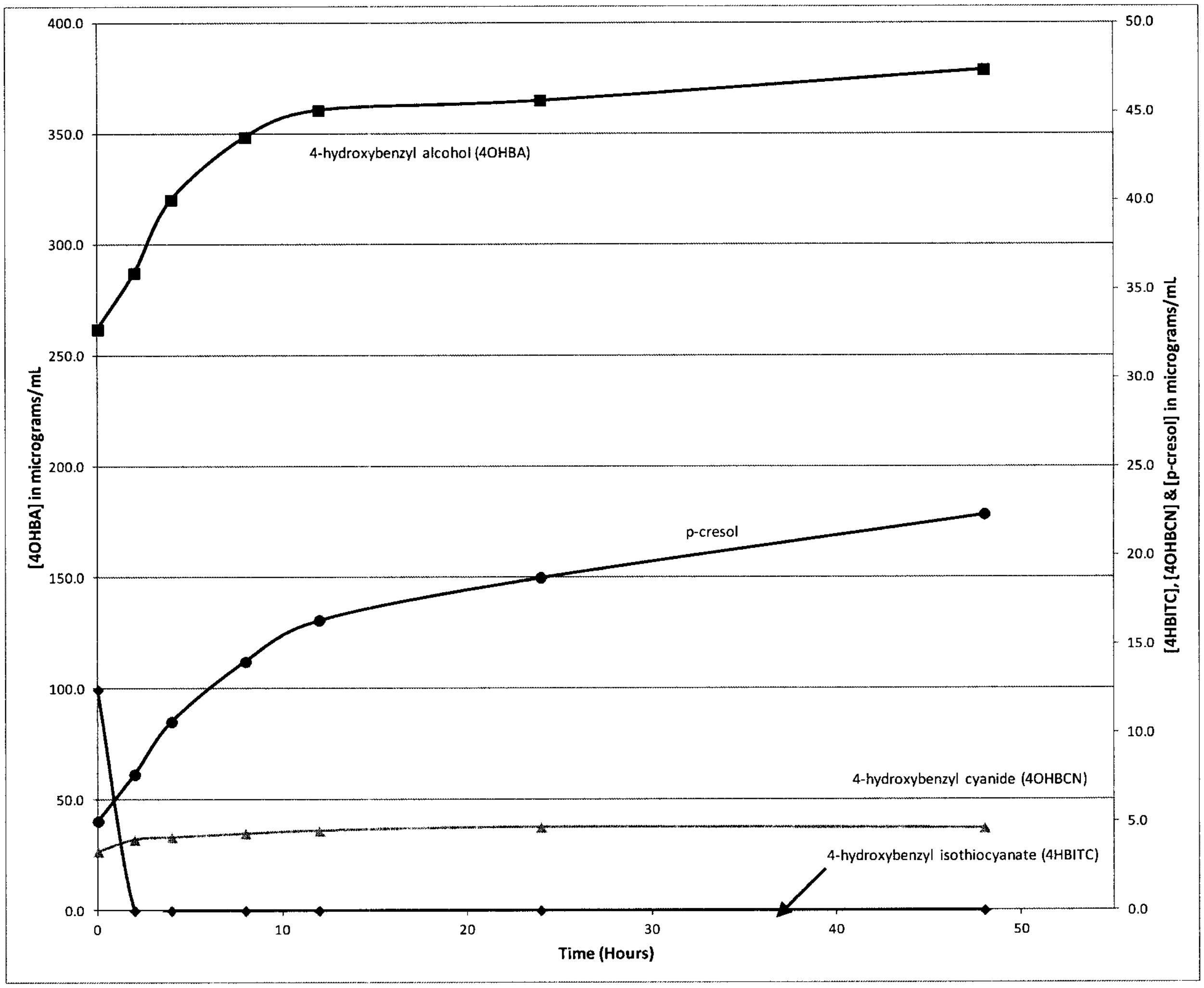
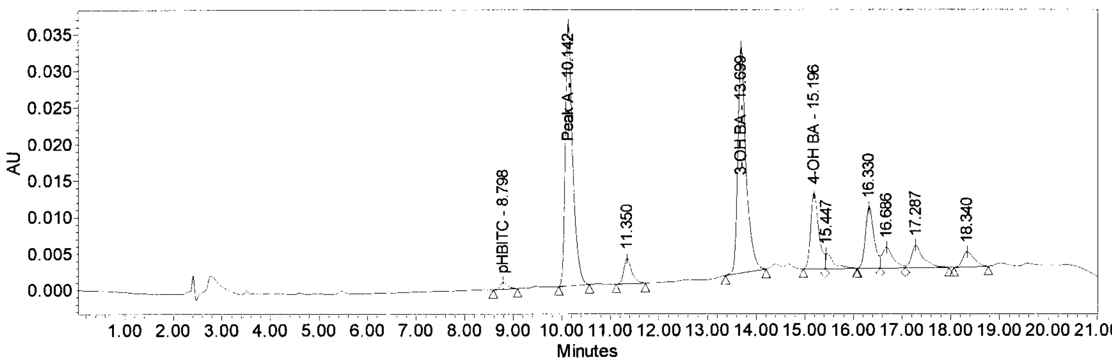
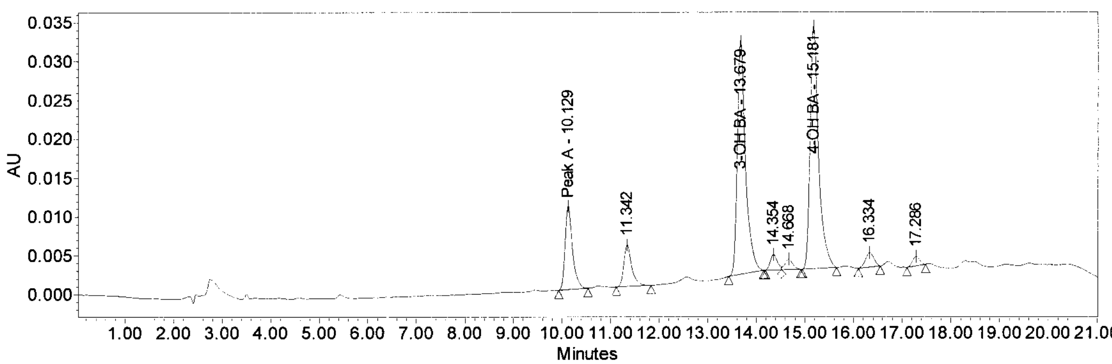


Figure 25. Normal phase chromatogram of the ethyl acetate extract for three time points for the pH 9 buffer. A.) 0 hours, B.) 8 hours, C.) 48 hours

A.) 0 hours



B.) 8 hours



C.) 48 hours

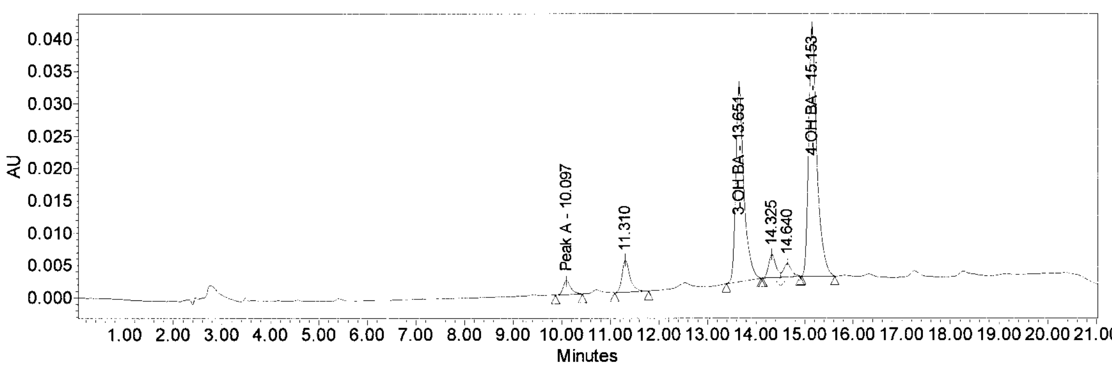


Figure 26. Disappearance of the compound represented by Peak A and the corresponding increase of 4OHBA during hydrolysis of 4HBITC at pH 9.0

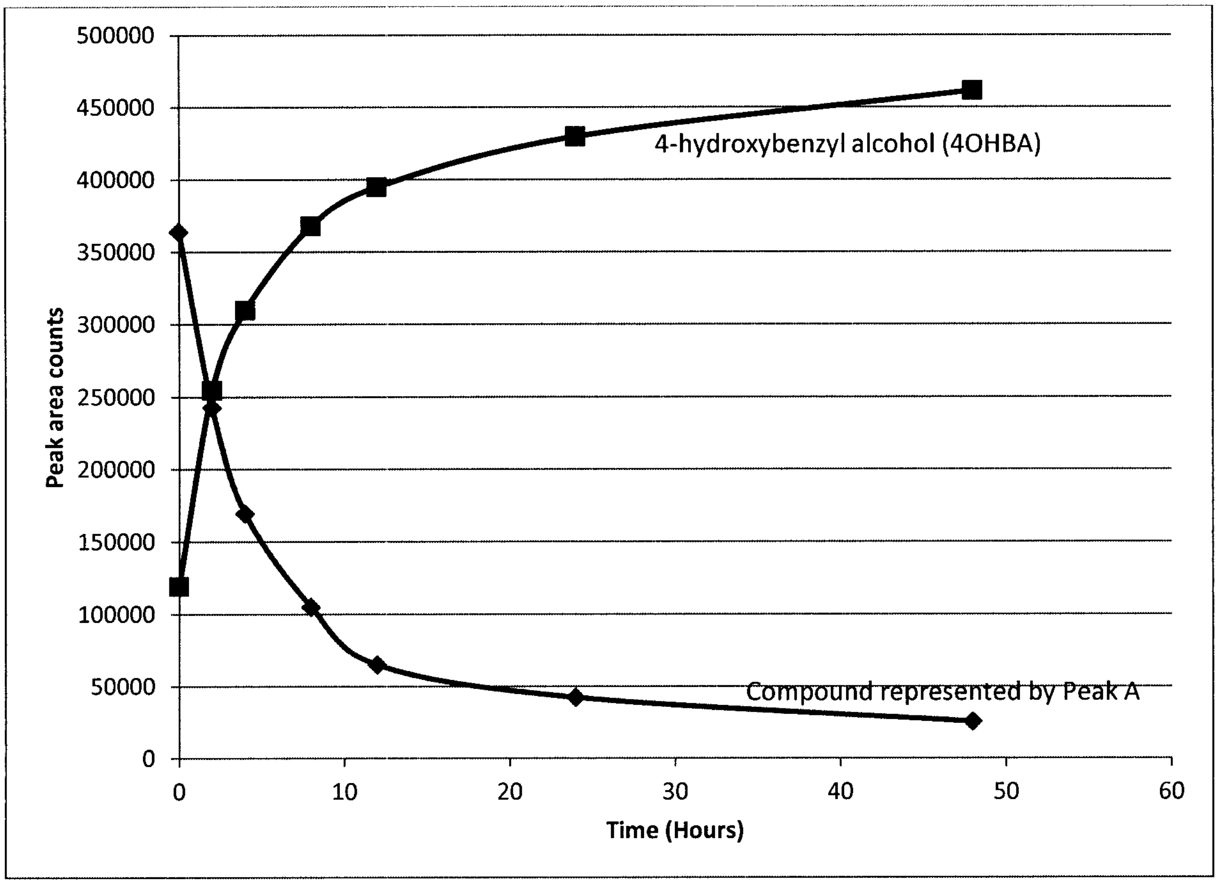


Figure 27. Concentration of 4HBITC in Roasted Red Bell Pepper puree, pH 4.97, 21°C. Average of 4 determinations for every time point.

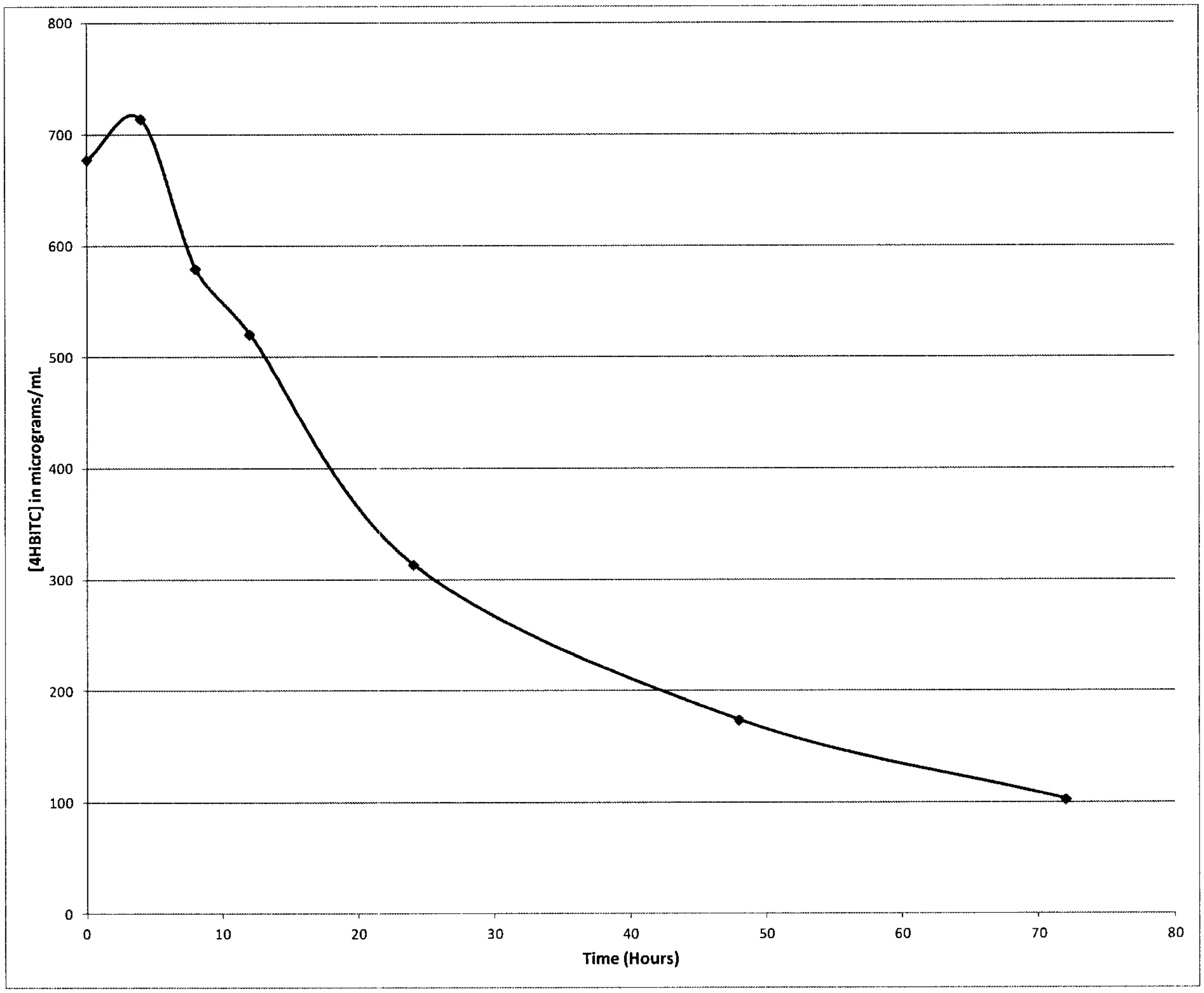
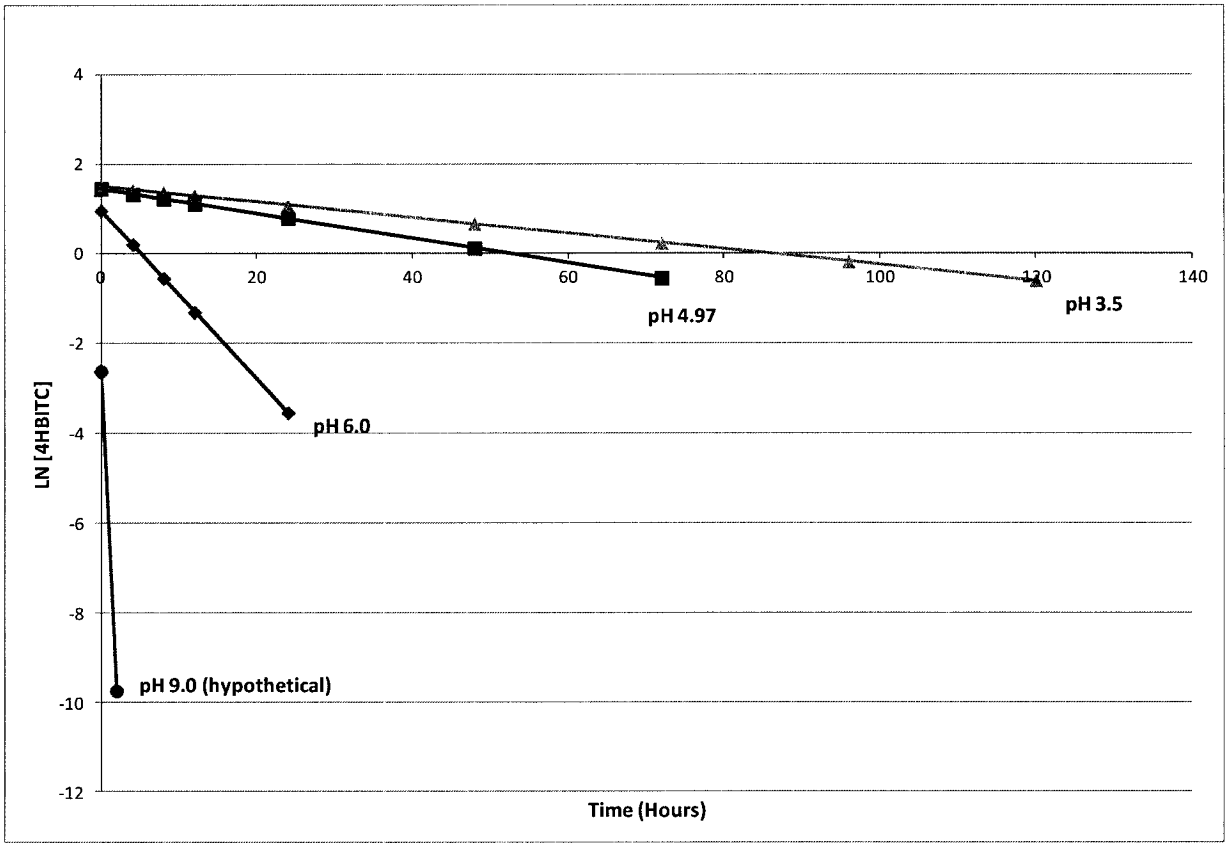


Figure 28. Comparison of the calculated regression lines at pH 3.5, pH 4.97 (Roasted Red Bell Pepper puree), pH 6.0 and pH 9.0 (hypothetical)



000118

Appendix – 1 Calculation of time to reach target final concentration.

Calculation of time to reach pre-set final concentration of 4HBITC using rate constants derived from hydrolysis experiments.

Assuming we will use pH 3.5 as the base rate equation for the hydrolysis of 4HBITC as this is the slowest rate of hydrolysis:

$$\mathrm{Ln}[4HBITC] = -0.017588 * \text{time} + 1.502754$$

This is in the form of $y = m*x + c$ where the independent variable x is time in hours. The constant c is the natural logarithm of the starting concentration in m mol/L (In this particular case we started with 1000 ppm added 4HBITC).

The starting concentration in m mol/L is equal to the concentration in ppm/molecular weight of 4HBITC which is 165.2043 Da.

As an example if we were starting with a [4HBITC] = 300 ppm

Then at time 0 hrs. Ln[4HBITC] = Ln[300/165.2043] = 0.5966

The rate equation will be Ln[4HBITC] = -0.017588 * time + 0.5966 ------------ Equation 1

If we were to find out the time required for the final [4HBITC] to be 10 ppm.

10 ppm = 10/165.2043 m mol/L = 0.060531 m mol/L

Ln[0.060531] = -2.8046. Substituting in the equation 1; time = (-2.8046-0.5966)/-0.017588

= 193 hrs.

= 8.0 days.

In 8 days a starting [4HBITC] = 300 ppm will be reduced to a final [4HBITC] = 10 ppm.

000119

Mass balance of 4HBITC hydrolysis products

Based upon literature and our own observations the primary non-sulfur degradation compound formed during 4-HBITC hydrolysis under all three pH values is 4-OHBA. We tracked the concentration of 4-OHBA, 4-OHBCN and p-cresol during the hydrolysis reactions. Based upon our data provided in Table 6 and that provided by Borek & Morra (2005), Kawakishi & Muramatsu (1966) & Kawakishi et al. (1967), we can say that the above three compounds and ionic thiocyanate (SCN^-) are the main degradation compounds resulting from the hydrolysis of 4-HBITC in aqueous systems. The HPLC chromatograms in the analytical section show numerous small peaks possibly indicative of several other natural compounds of very low concentration.

We can assume that the main hydrolysis reaction of 4HBITC is as follows:

$$\text{4-HBITC} + H_20 \longrightarrow \text{4OHBA} + SCN^- M^+$$

Here M+ is the cation/s from the medium.

Table 8. Mass balances of degradation compounds formed during the hydrolysis of 4-HBITC at different pHs'.

Starting concentration of 4HBITC is 1000 µg/mL or 6.05 m mol/L.

Buffer	Ending concentrations (m mol/L)				% 4HBITC
pH	4-HBITC	4-OHBA	4-OHBCN	p-cresol	Accounted for*
3.5	0.10	4.54	0.04	0.02	77.56
6.0	0.00	4.95	0.13	0.07	84.92
9.0	0.00	3.05	0.03	0.21	54.33

*without accounting for SCN^-

References:

1. Borek, V. and Morra, M.J. 2005. Ionic thiocyanate (SCN^-) production from 4-hydroxybenzyl glucosinolate contained in *Sinapis alba* seed meal. J. Agric. Food Chem., 53, 8650-8654.
2. Kawakishi, S. and Muramatsu, K. 1966. Studies on the decomposition of sinalbin Part I. The decomposition products of sinalbin. Agr. Biol. Chem., 30(7), 688-692.
3. Kawakishi, S., Namiki, M., Watanabe, H. and Muramatsu, K. 1967. Studies on the decomposition of sinalbin Part II. The decomposition products of sinalbin and their degradation pathways. Agr. Biol. Chem., 31(7), 823-830.

Appendix B

Appendix B. Certificate of Analysis (COA)

ConAgra Foods Food you love	**Certificate of Analysis**	Report Date: 6-29-2012

Sample ID: 237-11-736 (2,3,4,8)

Sample Name: WMEO Lot 1

Sample Description: White Mustard Essential Oil

Analysis:	**Result (%)**
4-Hydroxybenzylisothiocyanate	11.7%

Method Reference:
ENQA 6.1.A.5005 – Analysis of 4-Hydroxybenzylisothiocyate by HPLC.
(Sample is extracted in hexane-ethyl acetate (90:10 v/v) followed by HPLC analysis using gradient elution normal phase chromatography with UV detection at 277nm)

Test Location:
ConAgra Foods – Analytical Chemistry Laboratory
6 ConAgra Drive MS6-105
Omaha, NE 68102
402-240-6583

Report Reference:
60087-128-18

Report Released By:
Indarpal Singh – Senior Chemist

COVANCE

Report Number:	597347-0
Report Date:	13-Jul-2012
Report Status:	Final

Certificate of Analysis

Conagra Foods, Incorporated
6 ConAgra Drive, PDL - 105
Omaha Nebraska 68102 United States

Sample Name:	**White Mustard Essential Oil (WMEO Lot 1)**	**Covance Sample:**	**1446019**
Project ID	CONAGRA-20120706-0047	Receipt Date	03-Jul-2012
PO Number	Charge/VISA	Receipt Condition	5 (+/- 3) degrees Celsius
Sample Serving Size		Login Date	06-Jul-2012
Description	237-11-736 (2,3,4,8) 867261	Storage Condition	5 (+/- 3) degrees Celsius

Analysis	LOQ	Limit	Result	Pass/Fail
Residual Solvents				
Ethyl Acetate	20 ppm	5000 ppm	24.2 ppm	Pass

Method References	**Testing Location**
Residual Solvents (RESO_S:6)	Covance Laboratories Inc.

United States Pharmacopeia, 32nd Rev. - National Formulary 27th Ed., Method <467>, USP Convention, Inc., Rockville, MD (2009). (Modified).

Testing Location(s)	**Released on Behalf of Covance by**
Covance Laboratories Inc.	Lori Ross - Associate Director

Covance Laboratories Inc.
3301 Kinsman Blvd
Madison WI 53704
608-242-2712 x4170

ConAgra Foods
Food you love

Certificate of Analysis

Report Date: 6-29-2012

Sample ID: 237-11-736 (6,7,9,10)

Sample Name: WMEO Lot 2

Sample Description: White Mustard Essential Oil

Analysis:	**Result (%)**
4-Hydroxybenzylisothiocyanate	12.2%

Method Reference:

ENQA 6.1.A.5005 – Analysis of 4-Hydroxybenzylisothiocyate by HPLC. (Sample is extracted in hexane-ethyl acetate (90:10 v/v) followed by HPLC analysis using gradient elution normal phase chromatography with UV detection at 277nm)

Test Location:

ConAgra Foods – Analytical Chemistry Laboratory
6 ConAgra Drive MS6-105
Omaha, NE 68102
402-240-6583

Report Reference:
60087-128-16

Report Released By:
Indarpal Singh – Senior Chemist

COVANCE

Report Number: 597348-0
Report Date: 13-Jul-2012
Report Status: Final

Certificate of Analysis

Conagra Foods, Incorporated
6 ConAgra Drive, PDL - 105
Omaha Nebraska 68102 United States

Sample Name:	**White Mustard Essential Oil (WMEO Lot 2)**	**Covance Sample:**	**1446020**
Project ID	CONAGRA-20120706-0047	Receipt Date	03-Jul-2012
PO Number	Charge/VISA	Receipt Condition	5 (+/- 3) degrees Celsius
Sample Serving Size		Login Date	06-Jul-2012
Description	237-11-736 (6,7,9,10) 867261	Storage Condition	5 (+/- 3) degrees Celsius

Analysis	LOQ	Limit	Result	Pass/Fail
Residual Solvents				
Ethyl Acetate	20 ppm	5000 ppm	64.2 ppm	Pass

Method References	**Testing Location**
Residual Solvents (RESO_S:6)	Covance Laboratories Inc.

United States Pharmacopeia, 32nd Rev. - National Formulary 27th Ed., Method <467>, USP Convention, Inc., Rockville, MD (2009). (Modified).

Testing Location(s)	**Released on Behalf of Covance by**
Covance Laboratories Inc.	Lori Ross - Associate Director

3301 Kinsman Blvd
Madison WI 53704
608-242-2712 x4170

ConAgra Foods
Food you love

Certificate of Analysis

Report Date: 6-29-2012

Sample ID: 237-11-736 (11,12,13)

Sample Name: WMEO Lot 3

Sample Description: White Mustard Essential Oil

Analysis:	**Result (%)**
4-Hydroxybenzylisothiocyanate	10.8%

Method Reference:
ENQA 6.1.A.5005 – Analysis of 4-Hydroxybenzylisothiocyate by HPLC.
(Sample is extracted in hexane-ethyl acetate (90:10 v/v) followed by HPLC analysis using gradient elution normal phase chromatography with UV detection at 277nm)

Test Location:
ConAgra Foods – Analytical Chemistry Laboratory
6 ConAgra Drive MS6-105
Omaha, NE 68102
402-240-6583

Report Reference:
60087-128-17

Report Released By:
Indarpal Singh – Senior Chemist

COVANCE.

Report Number:	597349-0
Report Date:	13-Jul-2012
Report Status:	Final

Certificate of Analysis

Conagra Foods, Incorporated
6 ConAgra Drive, PDL - 105
Omaha Nebraska 68102 United States

Sample Name:	**White Mustard Essential Oil (WMEO Lot 3)**	**Covance Sample:**	**1446021**
Project ID	CONAGRA-20120706-0047	Receipt Date	03-Jul-2012
PO Number	Charge/VISA	Receipt Condition	5 (+/- 3) degrees Celsius
Sample Serving Size		Login Date	06-Jul-2012
Description	237-11-736 (11,12,13) 867261	Storage Condition	5 (+/- 3) degrees Celsius

Analysis	LOQ	Limit	Result	Pass/Fail
Residual Solvents				
Ethyl Acetate	20 ppm	5000 ppm	60.6 ppm	Pass

Method References — **Testing Location**

Residual Solvents (RESO_S:6) — Covance Laboratories Inc.

United States Pharmacopeia, 32nd Rev. - National Formulary 27th Ed., Method <467>, USP Convention, Inc., Rockville, MD (2009). (Modified).

Testing Location(s) — **Released on Behalf of Covance by**

Covance Laboratories Inc. — Lori Ross - Associate Director

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Madison WI 53704
608-242-2712 x4170

These results apply only to the items tested. This certificate of analysis shall not be reproduced, except in its entirety, without the written approval of Covance.

COVANCE.
3301 Kinsman Boulevard
Madison, WI 53704

Report Number:	**371346-0**
Report Date:	**13-Apr-2011**
Report Status:	**Final**

Certificate of Analysis

The Procter & Gamble Company

8700 Mason Montgomery Road
Mason Ohio 45040 United States

Sample Name:	**HCH-89-071.01**	**Covance Sample:**	**761093**
Project ID	PRCT_GMBLE-20110404-0087	**Receipt Date**	01-Apr-2011
PO Number	Charge MC	**Login Date**	04-Apr-2011
Description	white mustard essential oil 2007 crop year - has some ethyl acetate	**Storage Condition**	Ambient temperature
		Disposal Instruction	Dispose 30 days after final reported

Analysis/Result	**Result**
Elements by ICP Mass Spectrometry	
Antimony	<10.0 ppb
Arsenic	19.2 ppb
Bismuth	<10.0 ppb
Cadmium	<10.0 ppb
Copper	1290 ppb
Lead	30.1 ppb
Mercury	<10.0 ppb
Molybdenum	<10.0 ppb
Silver	<10.0 ppb
Tin by ICP Mass Spectrometry	
Tin	<10.0 ppb

Method References	**Testing Location**
Elements by ICP Mass Spectrometry (ICP_MS_S:11) Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA, Official Method 993.14 (Modified).	**Covance Laboratories Inc.**
Tin by ICP Mass Spectrometry (ICPMS_SN_S:1) Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA, Official Method 993.14 (Modified).	**Covance Laboratories Inc.**

Testing Location(s)

Covance Laboratories Inc.

3301 Kinsman Blvd
Madison WI 53704

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Lori Ross
Associate Director

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COVANCE
3301 Kinsman Boulevard
Madison, WI 53704

Report Number:	**371347-0**
Report Date:	**13-Apr-2011**
Report Status:	**Final**

Certificate of Analysis

The Procter & Gamble Company

8700 Mason Montgomery Road
Mason Ohio 45040 United States

Sample Name:	**HCH-89-071.03**	**Covance Sample:**	**761094**
Project ID	PRCT_GMBLE-20110404-0087	**Receipt Date**	01-Apr-2011
PO Number	Charge MC	**Login Date**	04-Apr-2011
Description	white mustard essential oil 2008 crop year - plated on maltodextrin	**Storage Condition**	Ambient temperature
		Disposal Instruction	Dispose 30 days after final reported

Analysis/Result	**Result**
Elements by ICP Mass Spectrometry	
Antimony	<10.0 ppb
Arsenic	10.0 ppb
Bismuth	<10.0 ppb
Cadmium	<10.0 ppb
Copper	168 ppb
Lead	<5.00 ppb
Mercury	<10.0 ppb
Molybdenum	11.9 ppb
Silver	<10.0 ppb
Tin by ICP Mass Spectrometry	
Tin	22.0 ppb

Method References	**Testing Location**
Elements by ICP Mass Spectrometry (ICP_MS_S:11)	**Covance Laboratories Inc.**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA, Official Method 993.14 (Modified)	
Tin by ICP Mass Spectrometry (ICPMS_SN_S:1)	**Covance Laboratories Inc.**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA, Official Method 993.14 (Modified).	

Testing Location(s)

Covance Laboratories Inc.

3301 Kinsman Blvd
Madison WI 53704

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COVANCE.
3301 Kinsman Boulevard
Madison, WI 53704

Report Number:	**371348-0**
Report Date:	**13-Apr-2011**
Report Status:	**Final**

Certificate of Analysis

The Procter & Gamble Company
8700 Mason Montgomery Road
Mason Ohio 45040 United States

Sample Name:	**HCH-89-071.05**	**Covance Sample:**	**761095**
Project ID	PRCT_GMBLE-20110404-0087	**Receipt Date**	01-Apr-2011
PO Number	Charge MC	**Login Date**	04-Apr-2011
Description	white mustard essential oil 2009 crop year - has some ethyl acetate	**Storage Condition**	Ambient temperature
		Disposal Instruction	Dispose 30 days after final reported

Analysis/Result	**Result**
Elements by ICP Mass Spectrometry	
Antimony	<10.0 ppb
Arsenic	16.0 ppb
Bismuth	<10.0 ppb
Cadmium	<10.0 ppb
Copper	1850 ppb
Lead	16.6 ppb
Mercury	<10.0 ppb
Molybdenum	<10.0 ppb
Silver	<10.0 ppb
Tin by ICP Mass Spectrometry	
Tin	17.2 ppb

Method References	**Testing Location**
Elements by ICP Mass Spectrometry (ICP_MS_S:11)	**Covance Laboratories Inc.**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA, Official Method 993.14 (Modified).	
Tin by ICP Mass Spectrometry (ICPMS_SN_S:1)	**Covance Laboratories Inc.**
Official Methods of Analysis of AOAC INTERNATIONAL, 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA, Official Method 993.14 (Modified).	

Testing Location(s)

Covance Laboratories Inc.

3301 Kinsman Blvd
Madison WI 53704

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Lori Ross

Associate Director

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COVANCE

Report Number:	**374884-0**
Report Date:	**21-Apr-2011**
Report Status:	**Final**

Certificate of Analysis

The Procter & Gamble Company
8700 Mason Montgomery Road
Mason Ohio 45040 United States

Sample Name:	**White Mustard Essential Oil**	**Covance Sample:**	**760589**
Project ID	PRCT_GMBLE-20110404-0085	**Receipt Date**	01-Apr-2011
PO Number	NA	**Receipt Condition**	5 (+/- 3) degrees Celsius
Description	HCH-89-071.01 2007 crop year has some ethyl acetate	**Login Date**	04-Apr-2011
		Storage Condition	5 (+/- 3) degrees Celsius

Analysis/Result	**Result**
Organochlorinated Pesticides	
Tecnazene	<125 ppb
HCB	<65.0 ppb
Alpha-BHC	<125 ppb
Propyzamide	<250 ppb
DCNA	<185 ppb
PCNB	<100 ppb
Gamma-BHC	<125 ppb
Beta-BHC	<125 ppb
Heptachlor	<125 ppb
Chlorothalonil	<125 ppb
Delta-BHC	<125 ppb
Vinclozolin	<250 ppb
Aldrin	<125 ppb
DCPA	<185 ppb
Heptachlor Epoxide	<125 ppb
Endosulfan I	<125 ppb
Dieldrin	<125 ppb
Captan	<500 ppb
Folpet	<315 ppb
p,p' - DDE	<125 ppb
Endrin	<185 ppb
Oxadiazon	<375 ppb
Endosulfan II	<185 ppb
p,p' - DDD	<185 ppb
p,p' - DDT	<250 ppb
Endosulfan Sulfate	<185 ppb
Captafol	<315 ppb
Dicofol	<315 ppb
Mirex	<125 ppb
Tetradifon	<185 ppb
Methoxychlor	<315 ppb
Cis-Permethrin	<213 ppb
Cypermethrin	<940 ppb

COVANCE.

Report Number:	**374884-0**
Report Date:	**21-Apr-2011**
Report Status:	**Final**

Certificate of Analysis

The Procter & Gamble Company

8700 Mason Montgomery Road
Mason Ohio 45040 United States

Sample Name:	**White Mustard Essential Oil**	**Covance Sample:**	**760589**
Project ID	PRCT_GMBLE-20110404-0085	**Receipt Date**	01-Apr-2011
PO Number	NA	**Receipt Condition**	5 (+/- 3) degrees Celsius
Description	HCH-89-071.01 2007 crop year has some ethyl acetate	**Login Date**	04-Apr-2011
		Storage Condition	5 (+/- 3) degrees Celsius

Analysis/Result	**Result**
Organochlorinated Pesticides	
Trans-Permethrin	<412 ppb

Analysis/Result	**Limit**	**Result**	**Pass/Fail**
USP Pesticides			
Pyrethrum (sum of cinerin I, cinerin II, jasmolin I, jasmolin II, pyrethrin I, and pyrethrin II)	3 mg/kg	<3.0 mg/kg	Pass

Method References	**Testing Location**
Organochlorinated Pesticides (OPCL_GRN_S:1)	**Covance Laboratories - Greenfield**

Hopper, M. L. and Griffitt, K. R., "Evaluation of an Automated Gel Permeation Cleanup and Evaporation Systems for Determining Pesticide Residues in Fatty Samples", Journal of the Association of Official Analytical Chemists, Vol. 70, No. 4, pp. 724-726 (1987) (Modified).

Pesticide Analytical Manual, Volume 1: Multiresidue Methods, 3rd Ed., Chapter 3, "Multiclass Multiresidue Methods: 304 Method for Fatty Foods", Food and Drug Administration, Washington, D.C. (1999) (Modified).

USP Pesticides (PS01_USP_S:2) — **Covance Laboratories - Greenfield**

Testing Location(s)

Covance Laboratories - Greenfield

671 S. Meridian Road
Greenfield IN 46140

Released on Behalf of Covance by

Sharon McKilligin
Associate Director

For questions on this report, please contact your Client Service Representative at 866-964-2034

COVANCE.

Report Number:	374885-0
Report Date:	21-Apr-2011
Report Status:	Final

Certificate of Analysis

The Procter & Gamble Company
8700 Mason Montgomery Road
Mason Ohio 45040 United States

Sample Name:	White Mustard Essential Oil	Covance Sample:	760590
Project ID	PRCT_GMBLE-20110404-0085	Receipt Date	01-Apr-2011
PO Number	NA	Receipt Condition	5 (+/- 3) degrees Celsius
Description	HCH-89-071.03 2008 crop year plated on maltodextrin	Login Date	04-Apr-2011
		Storage Condition	5 (+/- 3) degrees Celsius

Analysis/Result	Result
Organochlorinated Pesticides	
Tecnazene	<12.5 ppb
HCB	<6.50 ppb
Alpha-BHC	<12.5 ppb
Propyzamide	<25.0 ppb
DCNA	<18.5 ppb
PCNB	<10.0 ppb
Gamma-BHC	<12.5 ppb
Beta-BHC	<12.5 ppb
Heptachlor	<12.5 ppb
Chlorothalonil	<12.5 ppb
Delta-BHC	<12.5 ppb
Vinclozolin	<25.0 ppb
Aldrin	<12.5 ppb
DCPA	<18.5 ppb
Heptachlor Epoxide	<12.5 ppb
Endosulfan I	<12.5 ppb
Dieldrin	<12.5 ppb
Captan	<50.0 ppb
Folpet	<31.5 ppb
p,p' - DDE	<12.5 ppb
Endrin	<18.5 ppb
Oxadiazon	<37.5 ppb
Endosulfan II	<18.5 ppb
p,p' - DDD	<18.5 ppb
p,p' - DDT	<25.0 ppb
Endosulfan Sulfate	<18.5 ppb
Captafol	<31.5 ppb
Dicofol	<31.5 ppb
Mirex	<12.5 ppb
Tetradifon	<18.5 ppb
Methoxychlor	<31.5 ppb
Cis-Permethrin	<21.3 ppb
Cypermethrin	<94.0 ppb

COVANCE.

Report Number:	**374885-0**
Report Date:	**21-Apr-2011**
Report Status:	**Final**

Certificate of Analysis

The Procter & Gamble Company

8700 Mason Montgomery Road
Mason Ohio 45040 United States

Sample Name:	**White Mustard Essential Oil**	**Covance Sample:**	**760590**
Project ID	PRCT_GMBLE-20110404-0085	**Receipt Date**	01-Apr-2011
PO Number	NA	**Receipt Condition**	5 (+/- 3) degrees Celsius
Description	HCH-89-071.03 2008 crop year plated on maltodextrin	**Login Date** **Storage Condition**	04-Apr-2011 5 (+/- 3) degrees Celsius

Analysis/Result	**Result**
Organochlorinated Pesticides	
Trans-Permethrin	<41.2 ppb

Analysis/Result	**Limit**	**Result**	**Pass/Fail**
USP Pesticides			
Pyrethrum (sum of cinerin I, cinerin II, jasmolin I, jasmolin II, pyrethrin I, and pyrethrin II)	3 mg/kg	<3.0 mg/kg	Pass

Method References	**Testing Location**
Organochlorinated Pesticides (OPCL_GRN_S:1)	**Covance Laboratories - Greenfield**

Hopper, M. L. and Griffitt, K. R., "Evaluation of an Automated Gel Permeation Cleanup and Evaporation Systems for Determining Pesticide Residues in Fatty Samples", Journal of the Association of Official Analytical Chemists, Vol. 70, No. 4, pp. 724-726 (1987) (Modified).

Pesticide Analytical Manual, Volume 1: Multiresidue Methods, 3rd Ed., Chapter 3, "Multiclass Multiresidue Methods: 304 Method for Fatty Foods", Food and Drug Administration, Washington, D.C. (1999) (Modified).

USP Pesticides (PS01_USP_S:2) — **Covance Laboratories - Greenfield**

Testing Location(s)

Covance Laboratories - Greenfield

671 S. Meridian Road
Greenfield IN 46140

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Sharon McKilligin

Associate Director

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COVANCE.

Report Number:	**374886-0**
Report Date:	**21-Apr-2011**
Report Status:	**Final**

Certificate of Analysis

The Procter & Gamble Company
8700 Mason Montgomery Road
Mason Ohio 45040 United States

Sample Name:	**White Mustard Essential Oil**	**Covance Sample:**	**760591**
Project ID	PRCT_GMBLE-20110404-0085	**Receipt Date**	01-Apr-2011
PO Number	NA	**Receipt Condition**	5 (+/- 3) degrees Celsius
Description	HCH-89-071.05 2009 crop year has some ethyl acetate	**Login Date**	04-Apr-2011
		Storage Condition	5 (+/- 3) degrees Celsius

Analysis/Result	Result
Organochlorinated Pesticides	
Tecnazene	<125 ppb
HCB	<65.0 ppb
Alpha-BHC	<125 ppb
Propyzamide	<250 ppb
DCNA	<185 ppb
PCNB	<100 ppb
Gamma-BHC	<125 ppb
Beta-BHC	<125 ppb
Heptachlor	<125 ppb
Chlorothalonil	<125 ppb
Delta-BHC	<125 ppb
Vinclozolin	<250 ppb
Aldrin	<125 ppb
DCPA	<185 ppb
Heptachlor Epoxide	<125 ppb
Endosulfan I	<125 ppb
Dieldrin	<125 ppb
Captan	<500 ppb
Folpet	<315 ppb
p,p' - DDE	<125 ppb
Endrin	<185 ppb
Oxadiazon	<375 ppb
Endosulfan II	<185 ppb
p,p' - DDD	<185 ppb
p,p' - DDT	<250 ppb
Endosulfan Sulfate	<185 ppb
Captafol	<315 ppb
Dicofol	<315 ppb
Mirex	<125 ppb
Tetradifon	<185 ppb
Methoxychlor	<315 ppb
Cis-Permethrin	<213 ppb
Cypermethrin	<940 ppb

COVANCE.

Report Number:	**374886-0**
Report Date:	**21-Apr-2011**
Report Status:	**Final**

Certificate of Analysis

The Procter & Gamble Company

8700 Mason Montgomery Road
Mason Ohio 45040 United States

Sample Name:	**White Mustard Essential Oil**	**Covance Sample:**	**760591**
Project ID	PRCT_GMBLE-20110404-0085	**Receipt Date**	01-Apr-2011
PO Number	NA	**Receipt Condition**	5 (+/- 3) degrees Celsius
Description	HCH-89-071.05 2009 crop year has some ethyl acetate	**Login Date**	04-Apr-2011
		Storage Condition	5 (+/- 3) degrees Celsius

Analysis/Result	**Result**
Organochlorinated Pesticides	
Trans-Permethrin	<412 ppb

Analysis/Result	**Limit**	**Result**	**Pass/Fail**
USP Pesticides			
Pyrethrum (sum of cinerin I, cinerin II, jasmolin I, jasmolin II, pyrethrin I, and pyrethrin II)	3 mg/kg	<3.0 mg/kg	Pass

Method References	**Testing Location**
Organochlorinated Pesticides (OPCL_GRN_S:1)	**Covance Laboratories - Greenfield**

Hopper, M. L. and Griffitt, K. R., "Evaluation of an Automated Gel Permeation Cleanup and Evaporation Systems for Determining Pesticide Residues in Fatty Samples", Journal of the Association of Official Analytical Chemists, Vol. 70, No. 4, pp. 724-726 (1987) (Modified).

Pesticide Analytical Manual, Volume 1: Multiresidue Methods, 3rd Ed., Chapter 3, "Multiclass Multiresidue Methods: 304 Method for Fatty Foods", Food and Drug Administration, Washington, D.C. (1999) (Modified).

USP Pesticides (PS01_USP_S:2)	**Covance Laboratories - Greenfield**

Testing Location(s)

Covance Laboratories - Greenfield

671 S. Meridian Road
Greenfield IN 46140

Released on Behalf of Covance by

Sharon McKilligin
Associate Director

For questions on this report, please contact your Client Service Representative at 866-964-2034

Appendix C

Appendix C. Protein Characterization of WMEO

PROCTER & GAMBLE
TRACE ANALYTICAL CORE (TAC) MEMO

FROM:	Lijuan Li/Julie Hudia/Rohan Wimalasena	DATE:	10/18/2011
CC:	Darwin Popenoe		
TO:	Athula Ekanayake	R/L:	LOP + 3 Yrs

SUBJECT: Protein Characterization of White Mustard Essential Oil

PROJECT NAME OR NUMBER: Botanical-derived material-Protein Characterization

SUMMARY
The botanical-derived material, White Mustard Essential Oil (WMEO) was tested for protein content by amino acid analysis (AAA). Based on AAA results, the total protein (proteins and peptides) content in the material was determined to be 11.35 ppm. The extraction efficiency of proteins from WMEO was assumed to be 100%.

BACKGROUND
WMEO is being evaluated as potential natural preservative. Acquiring the necessary protein data to evaluate the safety of this material is critical to get FDA approval for the material.

WMEO (Notebook: HCH-89-090) was submitted for analysis for presence of proteins of molecular weight (MW) higher than 2.0 kD for safety evaluation. Since the material was manufactured from plant based materials (mustard seed) it is customary to verify the protein levels in these samples. Proteins of MW >2.0 kD are believed to have the potential to elicit allergenic reactions. Therefore, protein determination is critical to assure safety of the use of this material in consumer products.

EXPERIMENTAL
A single lot of WMEO was extracted in triplicate with ammonium bicarbonate to recover any proteins. Briefly, an aliquot (3.0 g) of WMEO was extracted with 3.0 mL of 0.2 M ammonium bicarbonate to selectively isolate the proteins from the oil. A 1-mL aliquot of the extract was dried under vacuum and submitted for AAA at W. M Keck Foundation, Yale University. Control samples were prepared along the WMEO samples following the same extraction procedure and were submitted for AAA as well.

ANALYTICAL RESULTS & DISCUSSION
Amino acid analysis results of WMEO under hydrolyzed and non-hydrolyzed conditions are summarized in Table 1. As shown in Table 1, the average amount of total proteins (proteins and peptides) in the triplicate analysis of WMEO is about 11.35 ppm after background subtraction. Since WMEO is unlikely to have any proteins based on the manufacturing process no additional work was done to establish the % recovery of proteins from WMEO. Potential protein levels in the samples were estimated assuming 100% recovery of proteins from the matrix.

Table 1. Total protein in WMEO by AAA

Sample ID	Hydrolyzed (ppm)	Non-hydrolyzed (ppm)	Total Proteins (Peptides and Proteins) (ppm)
WMEO extract 1	11.533	0.458	11.075
WMEO extract 2	12.058	0.469	11.589
WMEO extract 3	12.081	0.626	11.455
		Average	**11.373**
		STDEV	0.267
		%RSD	2.34
Control 1	0.016	Average of Control	**0.019**
Control 2	0.022		
WMEO background subtracted			**11.354**

CONCLUSION

WMEO has 11.35 ppm total protein (proteins and peptides) based on AAA data. The extraction efficiency of proteins from WMEO was assumed to be 100%.

Notebook Reference: MBL14582, CS 5

Appendix D

Appendix D. Efficacy Data to Support Intended Effect

Although mustard and other condiments and spices have been used as food preservatives since antiquity, the use of white or yellow mustard essential oil has been limited due possibly to its instability. White mustard essential oil (WMEO) and its active component 4-hydroxybenzyl isothiocyanate (4-HBITC) are not commercially available and as a result its antimicrobial properties have not yet been fully leveraged.

WMEO was first tested in an acidic (pH < 4) juice drink matrix against chemical preservative-resistant (potassium sorbate & sodium benzoate) spoilage organisms comprising three strains of *Gluconobacter* spp. and four strains of *Zygosaccharomyces bailii*. These are common juice and juice drink spoilage microorganisms. The more common method of processing high acid products is hot-fill-hold where the pasteurized juice is filled hot to pasteurize food contact surfaces and head space. This requires higher cost packaging material that can withstand heat treatment without deformation of plastic containers. Additionally there are losses in nutrients and quality factors during hot-fill-hold without instantaneous cooling. These disadvantages are overcome with the cold-fill-cap process. However cold-filled products are prone to recontamination with adventitious microorganisms. An assessment was needed to understand the role of WMEO as a secondary barrier for control of potential post-process recontamination during cold filling of acid products. A challenge study was conducted with 0, 12.5, 25 and 50 ppm 4-HBITC against a low inoculum level (15 cfu/mL) of mixed strains of *Gluconobacter* spp. and *Zygosaccharomyces bailii*, simulating sporadic post-process recontamination typically found in bottling lines. The product was preserved against these challenge microorganisms for a period of 4 weeks (test end point) stored at 22°C (Table 1).

Table D-1. Acidic Juice Drink with WMEO, Challenged with *Gluconobacter spp.* and *Z. bailii**

Plate Counts cfu/mL (Duplicate Plates)

[4-HBITC]	Week 1	Week 2	Week 3	Week 4
0 ppm	TNTC/TNTC**	-	-	-
12.5 ppm	TNTC/TNTC**	-	-	-
25 ppm	<1 / <1	<1 / <1	<1 / <1	<1 / <1
50 ppm	<1 / <1	<1 / <1	<1 / <1	<1 / <1

*This data has been published (Ekanayake et al., 2006).
**Too numerous to count

In calcium fortified (1.54 g L^{-1}) cold-filled juice drink, 80 ppm 4-HBITC was able to preserve the product against a similar post-process recontamination simulation as above. Divalent cations such as calcium are well known promoters of growth of spoilage microorganisms, namely *Gluconobacter* spp., and *Zygosaccharomyces bailii.* About 80 ppm 4-HBITC was able to control potential spoilage by these fastidious microorganisms despite calcium fortification of the juice drink, stored for a period of 4 weeks (test end point) at 22° C, and is shown in Table 2.

Table D-2. Calcium Fortified Acidic Juice Drink with WMEO, Challenged with *Gluconobacter spp.* and *Z. bailii*

Plate Counts cfu/mL (Duplicate Plates)

[4-HBITC]	Week 1	Week 2	Week 3	Week 4
0 ppm	TNTC/TNTC**	-	-	-
10 ppm	TNTC/TNTC**	-	-	-
19 ppm	TNTC/TNTC**	-	-	-
39 ppm	<1 / <1	<1 / <1	TNTC/TNTC	-
77 ppm	<1 / <1	<1 / <1	<1 / <1	<1 / <1

**Too numerous to count

In addition to the above specific in-product challenge studies, the following in-vitro broth studies were conducted to assess the antimicrobial effect of WMEO against Gram positive and Gram negative pathogens found in other non-acid or un-acidified products at neutral pH. Peptone broth (0.5% by weight, pH 7.1) was inoculated with *Escherichia coli (ATCC 35150), Salmonella enteritidis (ATCC 13076),* or *Listeria monocytogenes (ATCC 7644)* taken from 20-24 hour cultures grown on trypticase soy agar slants at 35°C. *Campylobacter jejuni (ATCC 29428)* cultures were grown on modified charcoal cefoperazone deoxycholate (MCCDA) agar (Hutchinson and Bolton, 1984) slants and incubated under microaerophilic conditions at 42°C. Organisms were harvested and suspended per standard microbiology laboratory procedures. Starting counts were ~ $1x10^7$ cfu mL^{-1}. WMEO in maltodextrin (stock concentration 2.4% 4-HBITC) was introduced into the inoculated media to reach starting 4-HBITC levels of 60, 120 and 360 ppm. Maltodexrin alone served as the negative control. Samples were incubated with shaking at 6.5°C and plated on microbial content test (MCT) agar at 1, 3 and 5 days after neutralization with Letheen and as per USP 26 (United States Pharmacopoeia, Rockville, MD, USA). In the following tables log reductions at 24 and 120 hours are shown, using the 0 ppm log count as the reference. The data in the following Table 3 were generated using raw data that was used to plot the graphs published earlier by Ekanayake, et al (2006).

Table D-3. In-vitro broth WMEO antimicrobial efficacy studies with *E. coli* O157:H7, *S. enteritidis, L. monocytogenes* and *C. jejuni* (**)

***E. coli* O157:H7**

	24 Hour		120 Hour	
[4-HBITC] (ppm)	Log count	Log reduction	Log count	Log reduction
0	7.62	0.08	7.51	0.18
60	6.21	1.49	5.60	2.10
120	6.15	1.56	4.93	2.77
360	3.15	4.56	1.00 *	6.51

* 1.0 log is the limit of detection

S. enteritidis

	24 Hour		120 Hour	
[4-HBITC] (ppm)	Log count	Log reduction	Log count	Log reduction
0	7.66	0.04	7.49	0.21
60	6.56	1.14	5.61	2.09
120	4.86	2.84	3.94	3.76
360	2.55	5.15	1.00 *	6.49

* 1.0 log is the limit of detection

L. monocytogenes

	24 Hour		120 Hour	
[4-HBITC] (ppm)	Log count	Log reduction	Log count	Log reduction
0	7.68	0.02	7.56	0.14
60	7.23	0.47	5.79	1.91
120	5.78	1.92	4.28	3.42
360	3.21	4.49	1.00 *	6.56

* 1.0 log is the limit of detection

C. jejuni

	24 Hour		120 Hour	
[4-HBITC] (ppm)	Log count	Log reduction	Log count	Log reduction
0	7.60	0.10	7.51	0.19
60	7.57	0.13	7.46	0.24
120	6.28	1.42	5.97	1.73
360	3.08	4.62	1.00 *	6.51

* 1.0 log is the limit of detection; ** Data has been published in graphical form (Ekanayake et al., 2006).

Further evaluation of the antimicrobial effect of WMEO was carried out against a series of food spoilage and pathogenic microorganisms in an in-vitro broth system (pH 5.0). WMEO was capable of inactivating spoilage organisms isolated from red pepper puree such as *Lactobacillus paracasei* subspecies paracasei (6 log reduction in 4 hrs), *Lactococcus plantarum* (6 log reduction in 4 hrs) and *Candida bombii* (5 log reduction in 4 hrs) in broth cultures at 360 ppm 4-HBITC. Also tomato sauce spoilage isolate *Lactobacillus fermentum* populations were reduced by < 3 log within 4 hours; and so also acidified sausage spoilage isolate *Lactobacillus fructivorans* by 1.5 log within 4 hours and by 4.0 log in 24 hours; and vegetative cells of *Bacillus cereus* by 2 log within 2 hours.

In roasted red bell pepper puree product (pH 5.0), a combination of a thermal treatment and WMEO was able to reduce cocktails of *Salmonella spp.* and *Listeria spp.* by 5 and < 3 log respectively, while having no effect against *Lactobacillus spp.* at a 4-HBITC concentration of 360 ppm as shown below in Table 4, Table 5 and Table 6 (ConAgra Laboratory data).

Table D-4. Effect of WMEO (360 ppm, 4-HBITC) and thermal treatment (150°F, 1 min.), followed by refrigerated storage (40°F) on *Salmonella* cocktail (*S. enteritidis, S. ganium, S. poona, S. senftenberg* and *S. tennessee*) in roasted red bell pepper puree

Salmonella spp. Log (cfu/mL)				
Time (hours)	**Control**	**WMEO alone**	**Heat alone**	**WMEO + Heat**
0	6.2	6.2	6.2	6.2
0.3	6.2	6.1	4.8	2.7
1	6.2	6.1	4.6	2.1
3	6.2	6.1	4.4	2.5
5	6.0	6.0	4.5	2.3
24	6.1	5.9	4.3	1.8
48	6.1	5.2	4.2	1.7
120	6.0	3.6	3.3	1.0

Table D-5. Effect of WMEO (360 ppm, 4-HBITC) and thermal treatment (150°F, 1 min.), followed by refrigerated storage (40°F) on *Listeria monocytogenes* (Scott A) in roasted red bell pepper puree

Listeria monocytogenes Log (cfu/mL)				
Time (hrs.)	**Control**	**WMEO**	**Heat**	**WMEO + Heat**
0	6.8	6.8	6.8	6.8
0.3	6.9	6.9	5.0	3.9
2	6.8	6.7	5.1	3.5
24	6.9	6.9	5.2	3.7
48	6.8	6.9	4.9	3.9

Table D-6. Effect of WMEO (360 ppm, 4-HBITC) and thermal treatment (150°F, 1 min.), followed by refrigerated storage (40°F) on cocktail of *Lactobacillus* spp. in roasted red bell pepper puree

Lactobacillus spp. Log (cfu/mL)				
Time (hrs.)	Control	WMEO	Heat	WMEO + heat
0	5.4	5.4	5.4	5.4
0.3	5.4	5.3	5.1	4.6
1	5.4	5.4	4.9	4.6
3	5.4	5.5	4.9	4.5
7	5.4	5.4	4.8	4.5
24	5.4	5.5	4.8	4.7
48	5.4	5.6	4.8	4.7

Exhibits

Exhibits

Exhibit 1. Report of the Expert Panel

EXPERT PANEL OPINION
THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF WHITE MUSTARD ESSENTIAL OIL (WMEO) FOR SELECT FOOD USES

Introduction

The undersigned, an independent panel of experts, qualified by their scientific training and national and international experience to evaluate the safety of food and food ingredients (the "Expert Panel"), was specially convened by ConAgra Foods, Inc., and asked to evaluate the safety and Generally Recognized as Safe (GRAS) status of the proposed use of white mustard essential oil (WMEO) for select food uses. WMEO is proposed for use as an antimicrobial in non-carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), ketchup, sauces/gravies in frozen meals, and egg substitutes (e.g., Egg Beaters). WMEO (containing 4-HBITC) is proposed to be added to deliver the following initial levels of 4-HBITC in foods: non-carbonated beverages, 25 ppm; ketchup, 150 ppm; sauces/gravies in frozen meals, 500 ppm; and egg substitutes (e.g., Egg Beaters), 250 ppm.

Exponent Inc. ("Exponent") performed a comprehensive search of the literature bearing on the safety of WMEO and its components and/or hydrolysis products in January 2012. The compounds relevant to a GRAS self-determination of the proposed uses of WMEO include the following hydrolysis products of sinalbin: 4-hydroxybenzyl isothiocyanate (4-HBITC, CAS #2086-86-4), 4-hydroxybenzyl alcohol (4-OHBA, CAS #623-05-2), 4-hydroxybenzyl cyanide (4-OHBCN, CAS #14191-95-8), and p-cresol (CAS #106-44-5). Exponent summarized the results of the literature search and prepared a safety dossier, "GRAS Self-Determination of White Mustard Essential Oil (WMEO) for Select Food Uses," for consideration by the Expert Panel.

The Expert Panel reviewed Exponent's safety documentation, as well as other available data and information that the Panel members believed to be pertinent to the safety of WMEO and its components/hydrolysis products under the conditions of intended use in certain food products. In addition, the Expert Panel reviewed the methods of manufacture and specifications for WMEO, analytical data confirming compliance with food-grade specifications, the conditions of intended uses in certain foods and the estimated dietary exposure to WMEO. The Expert Panel convened via telephone conference call, and independently, jointly, and unanimously concluded that WMEO, produced consistent with current good manufacturing practice (cGMP) and meeting appropriate specifications, is safe for its intended uses as listed in paragraph one above and under "Intended Use" below. The Expert Panel further concluded that these intended uses are GRAS based on scientific procedures. It is also the opinion of this Expert Panel that other qualified experts would concur with our conclusions.

Summarized below is the Panel's scientific analysis supporting our conclusions.

Description

White mustard essential oil (WMEO) is a natural antimicrobial that can be used to control potential pre- or post- process low level microbial contamination. Use of mustards as natural antimicrobials has been well established. Of the three varieties of mustard seeds, black (*Brassica nigra*), oriental (*Brassica juncea*), and white or yellow (*Sinapsis alba* or *Brassica hirta*) cultivated today, only white mustard contains the glucosinolate sinalbin almost exclusively. When white mustard seeds are ground and moistened, sinalbin is hydrolyzed to 4-hydroxybenzyl isothiocyanate (4-HBITC), a non-volatile, sharp-tasting oily compound.

4-HBITC has wide spectrum antimicrobial properties against bacteria, yeast and fungi at relatively low concentrations. It is also unstable in aqueous systems, hydrolyzing primarily to 4-hydroxybenzyl alcohol. The stability of 4-HBITC in aqueous systems is pH dependent and is more stable in acids (few days) and less stable with increasing pH, instantaneously decomposing at alkaline pH. Mass spectrometric analysis shows that the triglyceride fraction of the white mustard essential oil (WMEO) was identical to that of the cold pressed mustard oil and that the additional compounds found in WMEO are primarily 4-HBITC and other compounds of Brassica origin.

Mustard, white and yellow, are considered Generally Recognized As Safe (GRAS) by the U.S. Food and Drug Administration (FDA) for their intended use as spices, natural seasonings and flavorings per 21 CFR 182.10 and 21 CFR 582.10, and the essential oil of mustard is considered GRAS under 21 CFR 182.20 and 21 CFR 582.20. The component 4-hydroxybenzyl alcohol (4-

OHBA) that is formed from the hydrolysis and breakdown of sinalbin, is also listed as a GRAS flavoring substance by the Flavors and Extract Manufacturers' Association (FEMA; FEMA No. 3987). Similarly, the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2002) had no safety concerns for 4-OHBA at current levels of intake when used as a flavoring agent.

Manufacturing Process

WMEO is prepared by first cold pressing white mustard seeds to remove most of the fixed oil. The partially defatted mustard press cake is then moistened in the presence of ethyl acetate and the activator ascorbic acid. After the reaction period, the ethyl acetate extract containing 4-HBITC and the fixed oil in the starting partially defatted mustard press cake is removed by centrifugation. Low temperature evaporation of ethyl acetate under reduced pressure yields white mustard essential oil containing 4-HBITC and some of the fixed oil from the starting partially defatted mustard press cake. In order to stabilize the 4-HBITC in the WMEO and also provide a means of easily dispensing it, the WMEO is mixed with maltodextrin to provide an easily dispensable powdery material. It can also be diluted with a vegetable oil, dry powdered sugar or salt, for the same purpose. All of these carriers meet food grade specifications. Appropriate product specifications for WMEO have also been established.

Intended Use

WMEO is proposed to be added to non-carbonated beverages, ketchup, sauces/gravies in frozen meals, and egg beaters. WMEO (containing 4-HBITC) is proposed to be added to deliver the following initial levels of 4-HBITC in these specified foods: non-carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), 25 ppm; ketchup, 150 ppm; sauces/gravies in frozen meals, 500 ppm; and egg substitutes (e.g., Egg Beaters), 250 ppm.

Exposure

The consumption of non-carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), ketchup, sauces/gravies in frozen meals, and egg substitutes (e.g. Egg Beaters) was based on food consumption records collected as part of the National Health and Nutrition Examination Surveys (NHANES) conducted in 2003-2004 and 2005-2006.

The mean and 90th percentile estimates of food intake in g/kg-bw/day on both the *per capita* and *per user* bases were estimated. As would be expected, the non-carbonated beverages have the highest intake, with the energy, sport and fruit flavored drinks have the highest intake rate (13.68 g/kg bw/day at the 90th percentile *per user*) and juice drinks have the second highest intake rates (10.28 g/kg bw/day at the 90th percentile *per user*).

The mean and 90th percentile estimated daily intake (EDI) of 4-HBITC and its hydrolyzed metabolites based on the maximum proposed use levels of 4-HBITC in non-carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), ketchup, sauces/gravies in frozen meals, and egg substitutes (e.g. Egg Beaters) were estimated on both the *per capita* and *per user* bases. At the 90th percentile *per user*, the cumulative EDIs from all proposed food uses were 0.039, 0.21, 0.0019 and 0.0035 mg/kg bw/day for 4-HBITC, 4-OHBA, 4-OHBCN, and p-cresol, respectively.

Safety Data

Although complete safety data are not available for some of the components of WMEO, the publically available data that do exist combined with the widely disseminated knowledge concerning the chemistry of isothiocyanates and the long history of mustard consumption in general collectively provide a sufficient basis for an assessment of the safety of WMEO for the uses proposed herein. Safe exposure limits (ADI and/or background dietary exposure) for each of the compounds are as follows:

4-hydroxybenzyl isothiocyanate (4-HBITC)

A broadly based literature search revealed no toxicity information for 4-HBITC. However, some toxicity data were available for the closely related compound 4-BITC. The available Absorption Distribution Metabolism Excretion (ADME), short-term toxicity and mutagenicity data provide no basis for estimation of an ADI. Therefore, the background dietary exposure to white mustard seed, based on published information on the concentration of 4-HBITC in white mustard seed and mean per capita intakes of mustard seed in the U.S. of 1.2 g, allow an estimation of the daily intakes of 4-HBITC from mustard seed and range from approximately 14.4 to 40.8 mg (0.24 to

0.68 mg/kg/day for a 60 kg individual). The *per user* 90th percentile estimate of 0.039 mg/kg bw/day from the proposed uses is well below background dietary exposure to 4-HBITC.

4-hydroxybenzyl cyanide (4-OHBCN)

A broadly based literature search revealed only a subcutaneous LDLo in rats of 250 mg/kg. No other safety-related information was available. The *per user* 90th percentile estimate to 4-OHBCN from the proposed new uses of 4-HBITC is 0.0019 mg/kg bw/day is well below the background dietary intake of 4-OHBCN, which ranges from 2.3 to 6.4 mg (0.038 to 0.106 mg/kg/day for a 60 kg individual).

4-hydroxybenzyl alcohol (4-OHBA)

A broadly based literature search revealed only the results of an *in vitro* Ames assay in S. typhimurium strain TA100 (dose range 50-2000 µg/plate) with 4-HBA, the results of which were negative. No other safety-related information was available.

JECFA (2002) had no safety concerns for 4-OHBA at current levels of intake when used as a flavoring agent. As a flavoring agent, JECFA classified 4-OHBA as a structural class I. These classes of compounds were expected to be hydrolyzed to aromatic aldehydes and simple aliphatic alcohols. Based on the current intake from flavor uses, JECFA stated 4-OHBA had "no safety concern".

Based on published information on the concentration of 4-HBITC in white mustard seed and mean per capita intakes of mustard seed in the U.S. of 1.2 g, and conservatively assuming that all 4-HBITC formed converts entirely and exclusively to 4-OHBA, the estimated daily intake of 4-OHBA from mustard seed ranges from approximately 11.0 to 31.3 mg (0.18 to 0.52 mg/kg/day for a 60 kg individual). The per user 90th percentile estimate of 0.21 mg/kg bw/day from the proposed new uses is within the background dietary exposure. Given the inherent variability in the naturally occurring level of sinalbin in mustard, it is reasonable to assume that the cumulative exposure from the naturally occurring levels of 4-OHBA in the diet and the potential residual levels of 4-OHBA from the proposed use of WMEO would remain within the variable range of natural dietary exposure to 4-OHBA.

p-cresol

The potential toxicity of p-cresol has been studied and its safe use evaluated by government agencies and regulatory authorities such as the EPA, ATSDR, and RIVM. The available toxicology database consists of acute, subchronic, chronic and genotoxicity studies. A previously published chronic oral RfD for 4-methylphenol has been withdrawn by EPA as a result of agency review and a new RfD is in preparation. RIVM derived a TDI of 50 ug/kg bw/day (0.05 mg/kg bw/day) based on a marginal LOAEL of 50 mg/kg bw/day for CNS effects in a 13-week study rats and application of an uncertainty factor of 1000 (10 each to account for inter- and intraspecies variability and 10 for the lack of a NOAEL). ATSDR derived an intermediate oral MRL of 0.1 mg/kg bw/day based on the results of a subchronic study in male rats, and a chronic oral MRL based on results of a chronic (2-year) study in female mice (NTP, 2008) that was not available when RIVM evaluated p-cresol. NTP (2008) identified a LOAEL of 100 mg/kg bw/day for bronchiolar hyperplasia and thyroid follicular degeneration in female mice. Data from the NTP 13-week study were considered adequate for analysis using the benchmark dose approach for the intermediate oral MRL derivation. The male rat data set was selected for determining the point of departure for MRL derivation in order to be public health protective. Applying an uncertainty factor of 100 (10 for extrapolation from animals to humans and 10 for human variability) to the BMDL10 of 13.9 mg/kg bw/day yielded an intermediate-duration oral MRL of 0.1 mg/kg bw/day for *m/p*-cresol. ATSDR applied an uncertainty factor of 1000 (10 each for extrapolation from animals to humans, human variability, and use of LOAEL) to the LOAEL of 100 mg/kg bw/day for bronchiole hyperplasia of the lung and follicular degeneration of the thyroid gland in female mice for estimation of the chronic MRL of 0.1 mg/kg bw/day for *m/p*-cresol. The *per user* 90th percentile estimate of 0.0035 mg/kg bw/day from the proposed new uses is well below the intermediate and chronic oral MRLs of 0.1 mg/kg bw/day.

Summary

Our summary resulting from this critical evaluation is presented below.

- The substance that is the subject of this GRAS determination is white mustard essential oil (WMEO). WMEO, produced consistent with cGMP, meets appropriate specifications established by ConAgra Foods, Inc.

- Mustard, white and yellow, are considered Generally Recognized As Safe (GRAS) by the U.S. Food and Drug Administration (FDA) for their intended use as spices, natural seasonings and flavorings per 21 CFR 182.10 and 21 CFR 582.10, and the essential oil of mustard is considered GRAS under 21 CFR 182.20 and 21 CFR 582.20. The component 4-hydroxybenzyl alcohol (4-OHBA) that is formed from the hydrolysis and breakdown of sinalbin, is also listed as a GRAS flavoring substance by the Flavors and Extract Manufacturers' Association (FEMA; FEMA No. 3987). Similarly, the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2002) had no safety concerns for 4-OHBA at current levels of intake when used as a flavoring agent.

- WMEO is proposed for use as an antimicrobial and will be added to non-carbonated beverages (i.e., energy and sport drinks, fruit-flavored drinks, fruit juice, and juice drinks), ketchup, sauces/gravies in frozen meals, and egg substitutes – e.g., Egg Beaters. WMEO (containing 4-HBITC) is proposed to be added to deliver the following initial levels of 4-HBITC in foods: non-carbonated beverages, 25 ppm; ketchup, 150 ppm; sauces/gravies in frozen meals, 500 ppm; and egg substitutes – e.g., Egg Beaters, 250 ppm.

- The proposed use of WMEO provides 90th percentile estimated daily intakes (EDIs) for 4-HBITC, 4-OHBA, 4-HBCN, and p-cresol for the U.S. population (i.e., 0.0389, 0.21, 0.0019, and 0.0035 mg/kg bw/day, respectively) that are either below an established regulatory benchmark (e.g., p-cresol) or at or below the range of background dietary exposure to the compounds from consumption of mustard.

- Following a review of all available toxicity and exposure data for WMEO and its components, it can be concluded that the proposed use of WMEO is safe within the meaning of the FD&C Act, i.e., it meets the standard of reasonable certainty of no harm.

Expert Panel Conclusion

We, the undersigned expert panel members, have individually and collectively critically evaluated published and unpublished data and information pertinent to the safety of WMEO, and unanimously conclude that the use of WMEO, produced consistent with cGMP and meeting appropriate specifications, as an antimicrobial in select food uses as delineated above, is safe.

We further conclude that the intended use of WMEO produced consistent with cGMP and meeting appropriate specifications, as an antimicrobial in select food products as described above, is "generally recognized as safe" ("GRAS") based on scientific procedures.

It is our opinion that other qualified experts would concur with our conclusions.

By:

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SUBMISSION END